



School of Pharmacy and Pharmaceutical Sciences
Cardiff University
Cardiff

**Design, Synthesis and Biological Evaluation of Novel
anti-HCV Nucleosides and Nucleotides:
From Bench to the Clinical Trials**

A thesis submitted *in accordance with the conditions governing
candidates for the degree of*
Philosophiæ Doctor in Cardiff University

by
Karolina Weronika Madela

Supervisor: Prof. Christopher McGuigan

In collaboration with



July 2012

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of^{PhD}.....(insert MCh, MD, MPhil, PhD etc, as appropriate)

Signed...*Kandina Madela*.....(candidate) Date...*16.10.2012*.....

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references. The views expressed are my own.

Signed...*Kandina Madela*.....(candidate) Date...*16.10.2012*.....

STATEMENT 3: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Academic Standards & Quality Committee.**

Signed...*Kandina Madela*.....(candidate) Date...*16.10.2012*.....

ACKNOWLEDGEMENTS

First I would like to thank my supervisor Prof. Chris McGuigan, who gave me the opportunity to work within his excellent research team, for his support, patience and knowledge throughout my PhD, whilst allowing me to work in my own way. I could not wish for a better supervisor.

I would like to thank Inhibitex Inc., in Alpharetta (Atlanta) for funding my PhD, in particular I would like to thank Dr. Geoff Henson, Dr. Stan Chamberlain, Dr. Jeff Hutchins, Dr. John Vernachio and all their team for giving me a greater aim to my research.

My sincere thanks go to Ms. Helen Murphy who without fail, provided me with so many great things over the years. I thank her for all her help, constant assistance and patience(!), a friendly smile every day and unforgettable chats.

I would like to thank all past and present members of the group for making my time enjoyable and unforgettable.

My sincere thanks goes to all NMR managers (Marco, Rocco, Liv, Silvia, Maria and Michaela) for their time and effort in helping me with the overnight enzymatic assays.

I must thank Dr. Andrea Brancale for his help with molecular modeling studies.

I would also like to thank Dr. Mark Gumbleton and Dr. Matt Smith for performing the Caco-2 experiments.

Thanks to all technical staff for their invaluable support and assistance.

I would like to especially thank Fabrizio, Magda and Michaela for proofreading this work.

My very special thanks go to Magda and Rina. Their friendship, continuous encouragement, effort and time to listen to me (and my complaints) kept me on track. Without them this thesis would not have been completed.

Most of all I wish to thank my family, my mum, my sister, my niece and my grandparents for all their love, continuous support and encouragement they gave me over these years. It was so lovely to talk to you on the telephone, which always made me feel not too far away from home. Finally and most importantly I would like to thank Lukasz. You always took my problems as your own, helped me to overcome them, and encouraged me to achieve my goals. Thank you so much for your faithful love and endless help.

ABSTRACT

The search for new anti-HCV therapeutics continues, as current standard of care based on pegINF and ribavirin is limited and can cause significant side effects.

Several families of modified nucleoside are known to inhibit HCV RNA dependent RNA polymerase (RdRp). The 2'-C- β -methylguanosine (2'CMeG) has been identified as one of the most potent RdRp inhibitors ($IC_{50} = 0.26 \mu M$ as NTP). Despite its very good activity at the triphosphate level its poor cell uptake and presumably poor phosphorylation lead to significant decrease in active in HCV replicon assays ($EC_{50} = 3.5 \mu M$). The ProTide approach was applied to 2'-C-methylguanosine in order to by-pass the first phosphorylation limiting step and enhance cellular uptake. Modified nucleoside phosphoramidates with improved efficacy and selectivity may become a future of HCV therapies.

In the present work, the pronucleotide approach based on aryloxyphosphoramidate, phosphorodiamidate or oxazaphosphorine cyclic prodrugs has been applied to 2'-C- β -methylguanosine and related nucleoside analogues bearing modifications in C6-, C8- and C2- of the purine base. These modifications were mainly introduced to increase lipophilicity of 2'CMeG and consequently enhance cellular uptake and to deliver the 2'-C-methylguanosine 5'-monophosphate intracellularly.

In general, most of the newly synthesised compounds exhibited excellent potency in HCV replicon assay. The most potent compounds were up to 1000-fold more active than the corresponding parent nucleoside.

Base modification combined with the ProTide approach provide compounds that possess not only excellent antiviral activity but also good cell permeability. Extensive *in vitro* and *in vivo* studies lead to the selection of INX-08189 that has now progressed into human clinical trials for HCV, being currently in phase IIa clinical development.

PUBLICATIONS

A part of the work presented in this thesis has been published in the following journals:

1. McGuigan C, Perrone P, Madela K, Neyts J. The phosphoramidate ProTide approach greatly enhances the activity of β -2'-C-methylguanosine against hepatitis C virus. *Bioorg Med Chem Lett* **2009**, 19 (15), 4316-4320.
2. McGuigan C, Gilles A, Madela K, Aljarah M, Holl S, Jones S, Vernachio J, Hutchins J, Ames B, Bryant KD, Gorovits E, Ganguly B, Hunley D, Hall A, Kolykhalov A, Liu Y, Muhammad J, Raja N, Walters R, Wang J, Chamberlain S, Henson G. Phosphoramidate ProTides of 2'-C-methylguanosine as highly potent inhibitors of hepatitis C virus. Study of their in vitro and in vivo properties. *J Med Chem* **2010**, 53 (13), 4949-4957.
3. McGuigan C, Madela K, Aljarah M, Gilles A, Brancale A, Zonta N, Chamberlain S, Vernachio J, Hutchins J, Hall A, Ames B, Gorovits E, Ganguly B, Kolykhalov A, Wang J, Muhammad J, Patti JM, Henson G. Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus. *Bioorg Med Chem Lett* **2010**, 20 (16), 4850-4854.
4. Vernachio J, Bleiman B, Bryant KD, Chamberlain S, Hunley D, Hutchins J, Ames B, Gorovits E, Ganguly B, Hall A, Kolykhalov A, Liu Y, Muhammad J, Raja N, Walters CR, Wang J, Williams K, Patti JM, Henson G, Madela K, Aljarah M. INX-08189, a phosphoramidate prodrug of 6-O-methyl-2'-C-methyl guanosine, is a potent inhibitor of hepatitis C virus replication with excellent pharmacokinetic and pharmacodynamic properties *Antimicrobial Agents and Chemother* **2011**, 55 (5), 1843-1851.
5. McGuigan C, Madela K, Aljarah M, Gilles A, Battina SK, Ramamurty CVS, Rao CS, Vernachio J, Hutchins J, Hall A, Kolykhalov A, Henson G, Chamberlain S. Dual pro-drugs of 2'-C-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus. *Bioorg Med Chem Lett* **2011**, 21 (19), 6007-6012.
6. McGuigan C, Madela K, Aljarah M, Bourdin C, Arrica M, Barrett E, Jones S, Kolykhalov A, Bleiman B, Bryant KD, Ganguly B, Gorovits E, Henson G, Hunley D, Hutchins J, Muhammad J, Obikhod A, Patti JM, Walters CR, Wang J, Vernachio J, Ramamurty CVS, Battina SK, Chamberlain S. Phosphorodiamidates as a Promising

New Phosphate Prodrug Motif for Antiviral Drug Discovery: Application to Anti-HCV Agents. *J Med Chem* **2011**, 54 (24), 8632-8645.

7. Madela K, McGuigan C. Progress in the development of anti-HCV nucleoside and nucleotide prodrugs. *Future Med Chem* **2012**, 4(5), 625-650.

The full articles are referred in the Appendix I.

TABLE OF CONTENTS

Chapter One – Introduction

| | | |
|-------|--|----|
| 1.1 | The discovery of the Hepatitis C Virus (HCV) | 1 |
| 1.2 | HCV structure and genome organisation | 2 |
| 1.3 | HCV life cycle | 4 |
| 1.3.1 | Mechanism of viral entry into the host cell | 5 |
| 1.3.2 | HCV replication | 5 |
| 1.4 | Survival in the host | 6 |
| 1.5 | HCV genetic diversity | 7 |
| 1.6 | Vaccine development | 8 |
| 1.7 | Current treatment | 8 |
| 1.7.1 | Mechanism of action of therapeutic interferon | 10 |
| 1.7.2 | Mechanism of action of ribavirin | 10 |
| 1.7.3 | Boceprevir and Teleprevir – protease inhibitors | 13 |
| 1.8 | Polymerase inhibitors for the treatment of hepatitis C | 15 |

Chapter Two – Anti-HCV nucleos(t)ide prodrugs

| | | |
|---------|---|----|
| 2.1 | Prodrugs | 23 |
| 2.2 | Anti-HCV Nucleoside prodrugs in clinical trials | 24 |
| 2.2.1 | Valopicitabine (NM283) | 24 |
| 2.2.2 | Balapiravir (R1626) | 25 |
| 2.2.3 | Maricitabine (RG7128) | 26 |
| 2.3 | Nucleotide prodrugs – ProTides | 28 |
| 2.3.1 | Cyclosaligenyl (CycloSal) Phosphotriesters | 29 |
| 2.3.2 | SATE ProTides | 30 |
| 2.3.3 | POM approach | 30 |
| 2.3.4 | PAOB approach | 31 |
| 2.3.5 | Phosphoramidate di- and monoesters | 32 |
| 2.3.6 | Nucleoside phosphorodiamidates | 34 |
| 2.3.7 | Cyclic phosphoramidates | 34 |
| 2.3.8 | 3',5'-Cyclic ProTides | 35 |
| 2.3.9 | HepDirect prodrugs | 35 |
| 2.4 | Anti-HCV nucleotide prodrugs in clinical trials | 36 |
| 2.4.1 | PSI-7977 | 36 |
| 2.4.2.1 | IDX184 | 38 |
| 2.4.2.2 | INX-08189 | 39 |
| 2.4.2.3 | PSI-938 | 40 |
| 2.5 | Aims of the work | 42 |

Chapter Three – Phosphoramidate approach

| | | |
|---------|---|----|
| 3.1 | Aryl amino phosphoramidates – structure activity relationship studies | 55 |
| 3.2 | Synthesis | 56 |
| 3.2.1 | Preparation of phosphorochloridates | 57 |
| 3.2.1.1 | Synthesis of phosphorodichloridates | 57 |
| 3.2.1.2 | Synthesis of amino acid esters | 58 |
| 3.2.1.3 | Synthesis of phosphorochloridates | 60 |

| | | |
|-------|-------------------------------|----|
| 3.2.2 | Synthesis of phosphoramidates | 62 |
|-------|-------------------------------|----|

Chapter Four – Aryloxyphosphoramidates of 2'-C-β-methylguanosine.

| | | |
|--------|--|----|
| 4.1 | 2'-C-β-methylpurines | 65 |
| 4.2 | Synthesis of 2'-C-β-methylguanosine | 65 |
| 4.2.1 | Synthesis of 1,2,3,5-tetra- <i>O</i> -benzoyl-2'-C-methyl-D-ribofuranose (First method) | 66 |
| 4.2.2 | Synthesis of 1,2,3,5-tetra- <i>O</i> -benzoyl-2'-C-methyl-D-ribofuranose (Second method) | 67 |
| 4.2.3 | Coupling reaction | 67 |
| 4.3 | Synthesis of 2',3'-isopropylidene-2'-C-β-methylguanosine | 68 |
| 4.4 | Synthesis of 2'CMeG phosphoramidates | 69 |
| 4.5 | Biological evaluation of 2'-C-β-methylguanosine ProTides | 72 |
| 4.6 | Enzymatic studies using Carboxypeptidase Y on 2'CMeG ProTides | 77 |
| 4.7 | Synthesis of metabolic intermediates | 82 |
| 4.7.1 | Biological evaluation of phosphoramidate monoesters of 2'CMeG | 86 |
| 4.8 | Stability of 2'CMeG ProTides | 87 |
| 4.8.1 | Plasma stability | 87 |
| 4.8.2 | Liver and intestinal S9 stability | 89 |
| 4.8.3 | Stability in simulated gastric and intestinal fluids | 90 |
| 4.9 | Intracellular levels of 2'-C-methylguanosine triphosphate – <i>in vitro</i> studies | 92 |
| 4.9.1 | Intracellular metabolism in HCV replicon cells | 92 |
| 4.9.2 | Intracellular metabolism in primary human hepatocytes | 93 |
| 4.10 | Intracellular levels of 2'-C-methylguanosine triphosphate – <i>in vivo</i> studies | 94 |
| 4.10.1 | Intracellular levels of 2'-C-methylguanosine triphosphate in mouse model | 95 |
| 4.10.2 | Intracellular levels of 2'-C-methylguanosine triphosphate in nonhuman primate model | 97 |
| 4.11 | Conclusions | 98 |

Chapter Five – 2'-C-β-methylguanosine: new C-6 base modifications.

| | | |
|-------|--|-----|
| 5.1 | 6- <i>C</i> -modified 2'-C-β-methylguanosines – the rational behind the design | 103 |
| 5.2 | Synthesis of 6- <i>C</i> -modified 2'-C-β-methylguanosine analogues | 104 |
| 5.2.1 | Biological evaluation of 6- <i>C</i> -modified 2'-C-β-methylguanosine analogues | 107 |
| 5.2.3 | Adenosine deaminase assay on 6- <i>C</i> -modified 2'-C-β-methylguanosine analogues | 108 |
| 5.3 | Synthesis of 6- <i>C</i> -modified 2'-C-β-methylguanosine ProTides | 112 |
| 5.4 | Biological evaluation of first series of 6- <i>C</i> -modified 2'-C-β-methylguanosine ProTides | 114 |
| 5.5 | SAR studies of 6- <i>O</i> -methyl and 6- <i>O</i> -ethyl-2'-C-β-methylguanosine ProTides. | 116 |
| 5.5.1 | Synthesis and biological evaluation of 6- <i>O</i> -methyl-2'-C-β-methylguanosine phosphoramidates | 116 |

| | | |
|-------|---|------|
| 5.5.2 | Enzymatic studies using Carboxypeptidase Y on 6OMe2'CMeG ProTides | 1120 |
| 5.5.3 | Synthesis and biological evaluation of 6- <i>O</i> -ethyl-2'- <i>C</i> - β -methylguanosine phosphoramidates | 122 |
| 5.5.4 | Enzymatic and cell lysate studies on 6OEt2'CMeG ProTides | 124 |
| 5.5.5 | ProTides anti-HCV activity studies in the presence of adenosine and adenylate deaminase inhibitors | 126 |
| 5.5.6 | Effect of 6- <i>O</i> -alkylation on activity of 2'- <i>C</i> - β -methylguanosine phosphoramidates | 127 |
| 5.5.7 | Effect of 6- <i>O</i> -alkylation on membrane permeation | 128 |
| 5.6 | Intracellular levels of 2'- <i>C</i> -methylguanosine triphosphate – <i>in vitro</i> studies | 129 |
| 5.6.1 | Intracellular levels of 2'- <i>C</i> - β -methylguanosine triphosphate in replicon 1b cells – <i>in vitro</i> studies | 129 |
| 5.6.2 | Intracellular levels of 2'- <i>C</i> - β -methylguanosine triphosphate in human hepatocytes – <i>in vitro</i> studies | 130 |
| 5.7 | Conclusions | 132 |

Chapter Six – Identification of INX-189: from bench to the clinical trials.

| | | |
|-------|---|-----|
| 6.1 | Characterisation of INX-08189 | 137 |
| 6.2 | Biological evaluation and carboxypeptidase Y studies of separated isomers of INX-189 | 137 |
| 6.3 | Activity of INX-189 against wild type and resistant mutant HCV replicons | 140 |
| 6.3.1 | Anti-HCV activity of INX-189 against genotype 1a, 1b and 2a replicon cells | 140 |
| 6.3.2 | Rebound and clearance studies of INX-189 | 141 |
| 6.3.3 | Activity of INX-189 against resistant mutant strains | 143 |
| 6.4 | Intracellular levels of 2'- <i>C</i> - β -methylguanosine triphosphate – <i>in vivo</i> studies | 145 |
| 6.4.1 | Intracellular levels of 2'- <i>C</i> - β -methylguanosine triphosphate in rat model | 145 |
| 6.4.2 | Intracellular levels of 2'- <i>C</i> - β -methylguanosine triphosphate in cynomolgus monkey model | 146 |
| 6.5 | Results of the Phase Ia clinical trials of INX-189 | 148 |
| 6.6 | Results of the Phase Ib clinical trials of INX-189 | 148 |

Chapter Seven - New ProTide approach: Cyclic phosphoramidate (oxazaphosphorine) based prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine.

| | | |
|-------|---|-----|
| 7.1 | Cyclic phosphoramidates – rational behind the design | 153 |
| 7.2 | Synthesis of phosphorylating reagents | 154 |
| 7.2.1 | Synthesis of 2-Chloro-1,3,2-oxazaphosphinane 2-oxide | 154 |
| 7.2.2 | Synthesis of amino acid derived cyclic phosphorochloridates | 155 |
| 7.3 | Synthesis of oxazaphosphorine prodrugs of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 158 |
| 7.4 | Putative metabolic activation pathway | 159 |
| 7.4.1 | Human liver microsomes assay | 161 |

| | | |
|-----|---|-----|
| 7.5 | Biological evaluation of cyclic phosphoramidate prodrugs of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 162 |
| 7.6 | Conclusions | 163 |

Chapter Eight – Phosphorodiamidates - phosphate prodrugs.

| | | |
|-------|--|-----|
| 8.1 | Phosphorodiamidate prodrugs | 165 |
| 8.2 | Synthesis of symmetrical amino acid phosphorodiamidates of 6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 167 |
| 8.3 | Biological evaluation of symmetrical diamidates of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 169 |
| 8.4 | Enzymatic studies of symmetrical phosphorodiamidates of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 173 |
| 8.4.1 | Putative mechanism of activation of symmetrical diamidates | 174 |
| 8.4.2 | Carboxypeptidase Y studies of symmetrical diamidates | 175 |
| 8.4.3 | Huh7 cell lysate studies of symmetrical diamidates | 178 |
| 8.5 | Synthesis of asymmetrical phosphorodiamidates of 6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 179 |
| 8.5.1 | Synthesis of asymmetrical phosphorodiamidates via <i>p</i> -NO ₂ -phenyl route | 180 |
| 8.5.2 | Synthesis of asymmetrical phosphorodiamidates via phosphorodichloridate route | 181 |
| 8.5.3 | Synthesis of asymmetrical phosphorodiamidates via POCl ₃ route | 183 |
| 8.6 | Biological evaluation of asymmetrical diamidates of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 184 |
| 8.7 | Enzymatic studies of asymmetrical phosphorodiamidates of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine | 186 |
| 8.8 | Molecular modelling studies | 189 |
| 8.9 | Stability studies of 6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine symmetrical diamidates | 190 |
| 8.10 | Intracellular levels of 2'- <i>C</i> -methylguanosine triphosphate – <i>in vitro</i> studies | 192 |
| 8.11 | Intracellular levels of 2'- <i>C</i> -methylguanosine triphosphate – <i>in vivo</i> studies | 193 |
| 8.12 | <i>In vivo</i> toxicity studies | 194 |
| 8.13 | Conclusions | 195 |

Chapter Nine - In search of new pharmacophore: C-8 modifications.

| | | |
|-------|--|-----|
| 9.1 | 8- <i>C</i> -modified 6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine – the rational behind the design | 197 |
| 9.2 | Synthesis of 8- <i>C</i> -halo-6-modified-2'- <i>C</i> - β -methylguanosine nucleoside analogues | 197 |
| 9.2.1 | Synthesis of 8- <i>C</i> -bromo-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 197 |
| 9.2.2 | Synthesis of 8- <i>C</i> -chloro-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 198 |
| 9.2.3 | Synthesis of 8- <i>C</i> -iodo-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 199 |
| 9.2.4 | Biological evaluation of 8- <i>C</i> -halo-6-modified-2'- <i>C</i> - β -methylguanosine nucleoside analogues | 203 |

| | | |
|-------|---|-----|
| 9.2.5 | Adenosine deaminase assay on 8- <i>C</i> -iodo-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 204 |
| 9.3 | Synthesis of 8- <i>C</i> -halo-6- <i>O</i> -modified-2'- <i>C</i> - β -methylguanosine prodrugs | 205 |
| 9.3.1 | Synthesis of a first series of 8- <i>C</i> -halo-6- <i>O</i> -modified-2'- <i>C</i> - β -methylguanosine prodrugs | 205 |
| 9.3.2 | Biological evaluation of a first series of 8- <i>C</i> -halo-6- <i>O</i> -modified-2'- <i>C</i> - β -methylguanosine prodrugs | 208 |
| 9.3.3 | SAR studies of a second series of 8- <i>C</i> -iodo-6- <i>O</i> -modified-2'- <i>C</i> - β -methylguanosine prodrugs | 210 |
| 9.3.4 | Enzymatic studies in the presence of carboxypeptidase Y | 212 |
| 9.3.5 | Synthesis of metabolic intermediate 9.11 | 214 |
| 9.4 | Synthesis of a second series of 8-modified nucleosides | 216 |
| 9.4.1 | Synthesis of 8- <i>C</i> -methyl-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 217 |
| 9.4.2 | Synthesis of 8- <i>C</i> -phenyl-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 218 |
| 9.4.3 | Synthesis of 8- <i>C</i> -methoxy and 8- <i>C</i> -thiomethyl-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine | 218 |
| 9.4.4 | Biological evaluation of a second series of 8-modified 6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine nucleoside analogues | 219 |
| 9.5 | SAR of a second series of 8-modified 6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine ProTides | 220 |
| 9.6 | Understanding the 8- <i>C</i> -iodo phenomena. Analysis of hepatocyte metabolites of 8- <i>C</i> -iodo-6- <i>O</i> -methyl-2'- <i>C</i> -methylguanosine ProTides | 222 |
| 9.7 | Conclusions | 225 |

Chapter Ten - New family: C-2 modifications.

| | | |
|----------|---|-----|
| 10.1 | 2- <i>C</i> -modified-2'- <i>C</i> - β -methylguanosine – the rational behind the design | 229 |
| 10.2 | Synthesis of 2C-modified derivatives – 2C-benzylamine analogue | 229 |
| 10.2.1 | Synthesis of 2- <i>C</i> -benzylamine-2'- <i>C</i> - β -methylguanosine via diazotization-halogenation reaction | 230 |
| 10.2.2 | Synthesis of 2- <i>C</i> -benzylamine-2'- <i>C</i> - β -methylguanosine via convergent method | 231 |
| 10.2.2.1 | Synthesis of ProTides of 2- <i>C</i> -benzylamine analogues – first attempt | 232 |
| 10.2.2.2 | 2',3'-protection of 2- <i>C</i> -benzylamine-2'- <i>C</i> -methylguanosine | 233 |
| 10.2.3 | Synthesis of 2- <i>C</i> -benzylamine-6- <i>O</i> -methyl-2'- <i>C</i> - β -methylguanosine via halogenation reaction | 234 |
| 10.2.4 | Synthesis of ProTides of 2- <i>C</i> -benzylamine analogues | 236 |
| 10.3 | Synthesis of 2C-modified analogues – 2- <i>C</i> -thiophenyl analogues | 237 |
| 10.3.1 | Synthesis of ProTides of 2- <i>C</i> -thiophenyl analogues | 238 |
| 10.4 | Synthesis of 2C-modified analogues – 2- <i>C</i> -thiobenzyl analogues | 238 |
| 10.4.1 | Synthesis of ProTides of 2- <i>C</i> -thiobenzyl analogues | 239 |
| 10.5 | Synthesis of ProTides of other 2- <i>C</i> -modified analogues | 240 |
| 10.6 | Biological evaluation of 2- <i>C</i> -modified analogues and corresponding ProTides | 242 |
| 10.7 | Conclusions | 245 |

Chapter Eleven – Conclusions

| | | |
|------|-----------------------------------|-----|
| 11.1 | Main conclusions and perspectives | 249 |
|------|-----------------------------------|-----|

Chapter Twelve – Experimental part

| | | |
|------|--------------------------------------|-----|
| 12.1 | General experimental details | 251 |
| 12.2 | Standard procedures | 255 |
| 12.3 | Experimental Section – Chapter Three | 263 |
| 12.4 | Experimental Section – Chapter Four | 289 |
| 12.5 | Experimental Section – Chapter Five | 333 |
| 12.6 | Experimental Section – Chapter Seven | 373 |
| 12.7 | Experimental Section – Chapter Eight | 385 |
| 12.8 | Experimental Section – Chapter Nine | 413 |
| 12.9 | Experimental Section – Chapter Three | 451 |

| | |
|-------------------|------------|
| Appendix I | 473 |
|-------------------|------------|

ABBREVIATIONS AND ACRONYMS

| | |
|------------------|--|
| 2'CMeG | 2'-C-methylguanosine |
| 2'CMeGTP | 2'-C-methylguanosine triphosphate |
| 6OEt2'CMeG | 6-O-ethyl-2'-C-methylguanosine |
| 6OMe2'CMeG | 6-O-methyl-2'-C-methylguanosine |
| 5-FU | 5-fluorouracil |
| 5'-DP | 5'-diphosphate |
| 5'-MP | 5'-monophosphate |
| 5'-TP | 5'-triphosphate |
| α -Naph | α -naphthyl |
| AA | amino acid |
| Ac | acetyl |
| ACN | acetonitrile |
| ACV | acyclovir |
| ADA | adenosine deaminase |
| ADAL1 | adenosine deaminase-like protein 1 |
| ADME | absorption, distribution, metabolism and excretion |
| AMP | adenosine monophosphate |
| AMPDA | adenylate deaminase |
| Ar | aryl |
| AraG | 9- β -D-arabinofuranosylguanine |
| AUC | area under curve |
| AZT | 3'-azido-3'-deoxythymidine |
| AZU | 3'-azido-3'-deoxyuridine |
| BID | twice a day |
| BOC | <i>tert</i> -butyloxycarbonyl |
| Bz | benzoyl |
| BzCl | benzoyl chloride |
| cAMP | cyclic adenosine monophosphate |
| CatA | cathepsin A |
| CC ₅₀ | 50% cytotoxic concentration |

| | |
|------------------|---|
| cDNA | complementary DNA |
| ClogP | calculated logP |
| C _{max} | maximum concentration |
| Cpd | compound |
| CPY | carboxypeptidase Y |
| CTL | cytotoxic T lymphocytes |
| CycloSal | cyclosaligenyl triester |
| CYP | cytochrome P ₄₅₀ , isoenzyme |
| d4A | 2',3'-didehydro-2',3'-dideoxyadenosine |
| d4T | 2',3'-didehydro-2',3'-dideoxythymidine |
| DBU | 1,8-diazabicyclo[5.4.0]undec-7-ene |
| DCC | <i>N,N'</i> -dicyclohexylcarbodiimide |
| dCF | deoxycofymyrcine, pentostatin |
| dCK | deoxycytidine kinase |
| DCM | dichloromethane |
| dCMP | deoxycytidylate deaminase |
| ddA | 2',3'-dideoxyadenosine |
| ddI | 2',3'-dideoxyinosine |
| ddU | 2',3'-dideoxyuridine |
| DIPEA | diisopropylethylamine |
| DMA | <i>N,N</i> -dimethylacetamide |
| DMAP | 4-dimethylaminopyridine |
| DME | dimethoxyethane |
| DMF | dimethylformamide |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| D-PhG | D-phenylglycine |
| D-Val | D-valine |
| EC ₅₀ | 50% effective concentration |
| EC ₉₀ | 90% effective concentration |
| EHNA | erythro-9-(2-hydroxy-3-nonyl)adenine |
| EtOAc | ethyl acetate |

| | |
|---------------|---|
| EtOH | ethanol |
| FDA | Food and Drug Administration |
| FUdR | 5-fluoro-2'-deoxyuridine |
| hCE-1 | carboxylesterase 1 |
| HCV | hepatitis C virus |
| HINT1 | human histidine triad nucleotide binding protein 1 |
| HMDS | 1,1,1,3,3,3-hexamethyldisilazane |
| HPLC | high-performance liquid chromatography |
| HRMS | high resolution mass spectrometry |
| Huh 7 | human hepatoma cell line |
| HVR1 | hypervariable region 1 |
| IMPGH | inosine monophosphate dehydrogenase |
| INF | interferon |
| IRES | internal ribosome entry site |
| isoddA | |
| L-Ala | L-alanine |
| L-Ile | L-isoleucine |
| L-Met | L-methionine |
| L-Val | L-valine |
| LC-MS | liquid chromatography - mass spectrometry |
| <i>m</i> CPBA | <i>m</i> -chlorobenzoic acid |
| Me | methyl |
| MeMgBr | methylmagnesium bromide |
| MeOH | methanol |
| min | minutes |
| MS | mass spectrometry |
| MVA | modified virus of Ankara |
| NA | nucleoside analogue |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NANBH | non-A, non-B hepatitis |
| NBS | <i>N</i> -bromosuccinamide |
| NCS | <i>N</i> -chlorosuccinamide |

| | |
|--------------|--|
| NDPK | nucleoside diphosphate kinase |
| NI | nucleoside analogue inhibitor |
| NIS | <i>N</i> -iodosuccinamide |
| NK | natural killer cells |
| NMI | <i>N</i> -methylimidazole |
| NMR | nuclear magnetic resonance |
| NNI | non-nucleoside inhibitor |
| NTP | nucleoside triphosphate |
| OEt | ethoxy |
| OMe | methoxy |
| ORF | open reading frame |
| PAOB | <i>para</i> -acyloxybenzyl |
| PCV | penciclovir |
| PEG | polyethylene glycol |
| PENDANT | polarization enhancement during attached nucleus testing |
| Peg-INF | pegylated interferon |
| Ph | phenyl |
| PK | pharmacokinetic |
| PMEA | 9-[-2-(phosphonomethoxy)ethoxy]adenine |
| PMPA | 9-(R)-[2-(phosphonomethoxy)propyl]adenine |
| PO | <i>per os</i> / oral administration |
| POM | pivaloxymethyl |
| ProTide | pronucleotide, nucleotide prodrug |
| <i>p</i> TSA | <i>para</i> -toluenesulfonic acid monohydrate |
| QD | once a day |
| RBV | ribavirin |
| RDP | ribavirin diphosphate |
| RdRp | RNA dependant RNA polymerase |
| RNA | ribonucleic acid |
| RMP | ribavirin monophosphate |
| rt | room temperature |
| RTP | ribavirin triphosphate |

| | |
|-----------------|---|
| SATE | S-acyl-2-thioethyl |
| SGF | simulated gastric fluids |
| SIF | simulated intestinal fluids |
| SOC | standard of case |
| SVR | sustained viral response |
| <i>t</i> BuMgCl | <i>tert</i> -butylmagnesium chloride |
| Th1 | T helper cell |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| TMS | trimethylsilyl |
| TMSOTf | trimethylsilyl triflate |
| TP | triphosphate |
| TPPTS | 3,3',3''-phosphinidynetris(benzenesulfonic acid) trisodium salt |
| TRIZMA | 2-amino-2-hydroxymethylpropane-1,3-diol |
| UTR | untranslated region |
| VCD | vibrational circular dichroism |
| YMPK | uridine/cytidine monophosphate kinase |

CHAPTER ONE

Introduction

1.1 The discovery of the Hepatitis C Virus (HCV).

In the late 1930's it was believed that there are two different types of infectious jaundice.¹ These two types were distinguished by type of exposure and incubation period. Infectious hepatitis (type A) was characterised by relatively short incubation period, faecal-oral transmission and generally mild clinical course. Serum hepatitis (type B) had prolonged incubation period, parenteral transmission route and was more severe.¹

In mid 70's when serological tests for hepatitis B virus and later for hepatitis A virus were developed, transfusion related hepatitis studies were carried out.² During the studies several cases of an 'acute hepatitis' were identified. Surprisingly infected patients were lacking the typical hepatitis-like symptoms or jaundice and furthermore the incubation period differed from hepatitis A and B.^{2,3} It became clear that most of the transfusion-related hepatitis cases were due to neither of the previously known viruses, leading to the term non-A, non-B (NANB) hepatitis.² It was also discovered that approximately 10% of transfusions were shown to result in NANBH^{4,5}, which upon a lack of treatment caused persistent liver damage and could lead to liver cirrhosis.⁶ Interestingly, in some cases, the disease was occurring sporadically and was not related to the receipt of blood products.⁷ Regardless of ongoing progress in the research, all of the experiments failed to result in the molecular identification and isolation of the virus, antigen or antibody associated with NANBH. Furthermore, no NANBH- specific virus had been propagated *in vitro* or observed *in vivo*.⁸

In 1989, after 6 years of investigation at Chiron Corporation, a single cDNA clone (5-1-1) was isolated and was shown to encode an antigen specifically associated with NANB hepatitis. The clone was identified as a flavi-like virus, named hepatitis C virus (HCV).^{8,9} Development of specific HCV antibody immunoassays confirmed that as many as 70-90% of NANBH cases were associated with hepatitis C virus infection.¹⁰ The isolation of clone 5-1-1 allowed the whole viral genome to be identified and sequenced. Subsequently, new blood screening tests enabled detection of circulating HCV antibodies and viral RNA particles, effectively eradicating the

transmission of transfusion related NANBH.⁸ Currently the main routes of HCV transmission are intravenous drug abuse and modern medical practice.¹¹

1.2 HCV structure and genome organisation.

HCV is a small, enveloped virus with a single stranded (+) RNA genome. It belongs to the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome is approximately 9600 base pairs long, contains 5' and 3' untranslated (noncoding) regions and a single, open reading frame (ORF) encoding a polyprotein (~3000 amino acids) that is cleaved by viral and cellular proteases into 3 structural (core, E1 and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Figure 1.1).¹²

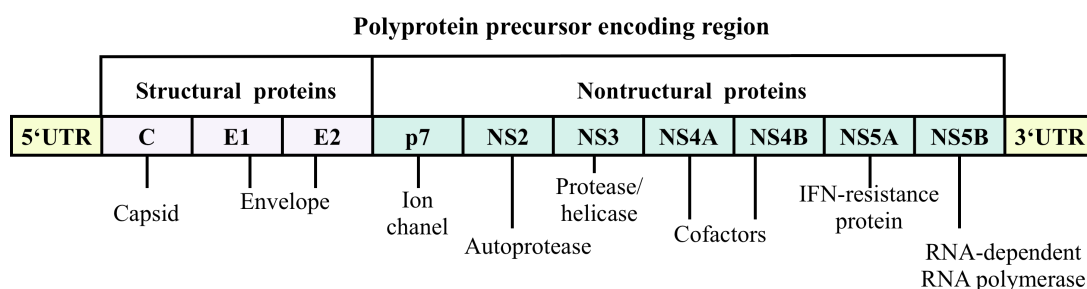


Figure 1.1. Organization of HCV genome.

5' and 3' untranslated regions (5'UTR and 3'UTR) are the most conserved regions of viral RNA.¹³ Up to 90% of the 5'UTR primary sequence is identical across the different HCV genotypes. 5'UTR consist of four domains, three of which are building the internal ribosome entry site, critical for initiation of viral RNA translation. The 3'UTR region contains in its primary sequence the termination codon of the HCV polyprotein.^{13,14}

The core protein is derived from the N-terminus of the polyprotein. It is highly conserved protein. It contains three domains:

- The *N*-terminal very hydrophilic domain (D1) is involved in RNA binding, nuclear localization and mediates capsid assembly.
- The *C*-terminal hydrophobic domain (D2) is responsible for association of the core protein with lipids and cellular membrane proteins.^{12,13}

The main function of the core protein is formation of the viral nucleocapsid, furthermore the core protein is involved in several regulatory functions including modulation of host signalling pathways, cellular and viral genome expression and cell

apoptosis. After translation, core protein is cleaved from the polyprotein by host enzymes.^{12,15}

E1 and E2 are glycoprotein components of the envelope, essential for viral fusion with host cell membrane, entry and also viral particle assembly. E2 consists of hypervariable region 1 (HVR1). HVR1 is a basic region with positively charged residues that can interact with negatively charged proteins at the cell surface. Therefore E1 can play a crucial role in host cell recognition and attachment, as well as cellular compartmentalization of the virus. E1 and E2 are cleaved from the polyprotein by host proteases.^{12,16}

p7 is a small polypeptide responsible for the production of infected particles *in vivo*.¹⁷ It is thought that p7 can mediate membrane permeability by formation of an ion channel (calcium ion channel) in the cell membrane acting as a viroporin. It is also believed that p7 can play an important role in viral particle release and maturation. p7 is cleaved from the polyprotein by host proteases.^{12,18}

NS2 is transmembrane protein responsible for endoplasmic reticulum membrane association; together with NS3 it shows autoprotease activity. After intramolecular cleavage from NS3, NS2 loses its protease activity and after phosphorylation undergoes degradation by the proteasome.^{12,19}

NS3 is a multifunctional protein. One third of its N-terminal region contains trypsin/chymotrypsin-like serine protease domain (NS3-4A) and RNA helicase domain (NS3) in the remaining C-terminal part of the polypeptide.¹² The NS3 binds non-covalently to NS4A protein, which acts as an activating cofactor.²⁰ NS4A increases the enzymatic activity of the NS3. NS3-4A is essential for the cleavage of the remaining downstream cleavages of the HCV polyprotein, into four functional non-structural proteins. NS4A stabilizes the N-terminal domain of the protein, by optimizing the orientation of the residues of the catalytic triad and by participating in formation of substrate recognition site.²⁰ Active NS3-4A protease is also thought to prevent interferon (INF) and pro-inflammatory cytokines induced antiviral response and therefore enables survival of HCV in the host.¹²

The C-terminal part possesses ATPase/helicase activity. It is thought that the main role lies in unwinding of the double stranded RNA intermediate, which is formed when the single HCV genome is replicated. However the precise role of the HCV helicase is not yet fully understood.¹²

NS4B is an ER membrane-associated protein. It is responsible for the formation of a special membrane compartments: membranous web and speckle-like structures that may serve as a platform for viral RNA replication. Additional presumed properties include modulation of NS5B RNA-dependant RNA polymerase activity, inhibition of cellular synthases and induction of interleukins and inflammatory responses.^{12,21}

NS5A is an anchored in membrane protein, responsible for regulation of RNA replication by interactions with cellular and viral proteins. NS5A protein exists in two forms: phosphorylated and hyperphosphorylated, each of them play different roles in the HCV life cycle. Phosphorylation of NS5A regulates a switch from replication to viral capsid assembly. The hyperphosphorylated form of the protein is thought to be responsible for the down-regulation of HCV replication. NS5A is also considered as an agent modulating the response to interferon based therapy.^{12,22,23}

NS5B is a 68kDa protein acting as a RNA-dependant RNA polymerase (RdRp) that lacks proofreading function. It consists of typical ‘palm’, ‘fingers’ and ‘thumb’ domains.²⁴ Interactions between domains result in fully enclosed active site into which nucleoside triphosphates can get access through a specific tunnel.²⁵ Two critical aspartic acid residues are located in the palm domain of an active site and are responsible for chelating metal ions (Mg^{2+} , Mn^{2+}) that are responsible for the polymerization reaction. Additionally, they coordinate the attack of the 3'-hydroxyl group of the growing RNA chain on the alpha-phosphate of the incoming nucleotide.^{24,25} Replication of the viral RNA has been found to proceed in the absence of primer (*de novo* synthesis). RdRp is an error-prone enzyme, responsible for the high degree of genetic variability.^{26,27}

1.3 HCV life cycle.

There are some key steps in HCV life cycle: entry to the host cell, viral genome uncoating, translation and replication of the viral genome, virion assembly and release. Each of these steps may present suitable target for therapeutic intervention.

1.3.1 Mechanism of viral entry into the host cell.

There are two suggested models of HCV cell entry. In the first one, virus entry is caused by direct interaction of envelope glycoproteins with specific host cell receptor complex SR-BI and CD81. Virus bound to the CD81 is transferred to the tight junctions where it interacts with claudin 1 and occludin. HCV enters the cell by clathrin-dependent endocytosis. Acidification of early endosome leads to the fusion of the viral envelope and release of nucleocapsid into the cytosol.^{28,29}

In the second mechanism, binding and internalization of the virus is mediated by interaction between HCV-associated lipoproteins with receptors on a cell surface. After attachment to the SR-BI-CD81 complex, the virus is subsequently transferred to the tight junction proteins. Then it undergoes mediated by envelope glycoproteins endocytosis and fusion. The last one leads to the release of viral genome from the early endosome.^{28,29}

1.3.2 HCV replication.

Decapsidation of viral nucleocapsid liberates free genomic RNA into the host cell. After exposure to the host cell machinery, it undergoes translation. As already mentioned 5'UTR region of HCV genome contains an internal ribosome entry site (IRES) that allows access of the viral genome to the host translation system. Translation of the HCV genome gives a single ~ 3000 amino acid polypeptide, which is processed by cellular and viral proteases to give structural and non-structural viral proteins. Translation of the viral proteins is necessary to establish the viral replication machinery.¹²

HCV is believed to replicate in association with intercellular membranes. It is suggested that the intercellular membrane can provide physical support, enabling a high local concentration of viral factors for its replication, and it facilitates the structural organization of the replication complex.³⁰

The positive sense RNA serves as a template for the synthesis of single, negative-strand RNA. The two RNA strands remained base-paired, however it is not clear when this putative complex is unwinded. The newly synthesised (-) RNA strand is copied multiple times by semiconservative replication mediated by RNA dependant RNA polymerase (RdRp). As a result, multiple positive-strand RNA genomes are

obtained. These particles can be either encapsulated to form new viruses or used as templates for viral protein synthesis. The newly formed viral particles are secreted from the cell after passage through the Golgi apparatus by exocytosis (Figure 1.2).^{12,14,31}

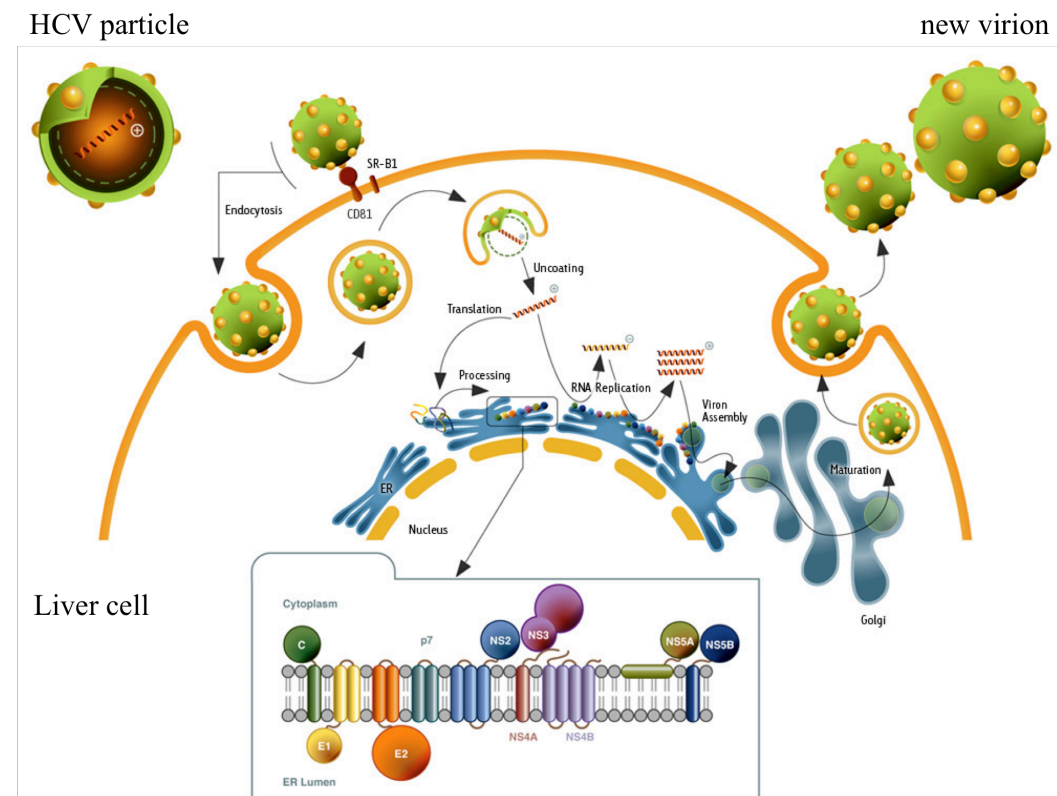


Figure 1.2. HCV replication.³²

1.4 Survival in the host.

The ease with which hepatitis C virus can persist within a host cells is astounding. This is mainly attributed to its capability to avoid the components of the adaptive and innate host immune system.

Host cells have developed a number of mechanisms to stop usage of their own translation complexes as an antiviral strategy. For example, in response to viral infection host cap-dependant translation can be shut down. Viral IRES that permits ready access of the viral genome to the translation machinery is cap-independent and enables viral translation to continue.³³

The key sites of HCV control over the host response are found to be related with:

- Reduction in IFN production in hepatocytes

- Suppression of cytokines signalling
- Inhibition of NK activity
- The INF-responsiveness of cellular factors is decreased
- Inability of the adaptive immune system to clear viral infection
- Enhanced permissiveness of HCV infection leading to the broader systemic immune defects (lack of antibody and CD8+T cell recognition).³³⁻³⁵

1.5 HCV genetic diversity.

As was already mentioned, NS5B encodes RNA-dependant RNA polymerase that lacks proofreading function. With a mean frequency of nucleotide substitutions of $1.4-1.9 \times 10^3$ substitution per site per year, very high production of virions per day and turn-over rate close to 99%, HCV can produce every possible mutation in every position each day.³⁶ This results in a elevated generation of genetic diversity, such that the virus exists as quasispecies, which is a collection of closely related but not identical genomes.³⁶ Based on phylogenetic analysis, HCV has been classified into six major genotypes (represented by numerous subtypes). Genotypes can differ from each other by ~30% and by less than 10% between subtypes. It has been suggested that potential endemic regions for the different genotypes are Guinea coast of West Africa for type 1, Western and Central Africa for genotype 2, Northern Indian subcontinent for type 3, Central Africa – 4, South-East Asia for type 6 (Table 1.1). The provenance of the genotype 5 is still under investigation.³⁷

Genotype distribution:

| Genotype | Area of distribution |
|----------|---|
| 1, 2, 3 | USA, Europe, Australia, East Asia (Japan, Taiwan, Thailand and China) |
| 4 | Middle East, Egypt and Central Africa |
| 5 | South Africa |
| 6 | South-East Asia and Australia |

Table 1.1. HCV genotype distribution.

HCV genotypes are very closely associated with the response to antiviral therapy. Therefore, further understanding of the clinical relevance and therapeutic

implications of the HCV genotype is crucial for designing individualized therapies for patients with HCV infection.³⁷

1.6 Vaccine development

There is no vaccine available for HCV infection. Lack of cell culture HCV (HCV could be generated *in vitro* in tissue culture systems only very recently) and proper animal models have greatly limited the development of HCV vaccines. Presently, various vaccines are primarily tested in chimpanzees. One of the biggest challenges for the HCV vaccines is fact that they will have to deal with high levels of viral genetic diversity and be able to eradicate HCV from the liver without inducing liver immunopathology. Most of the vaccines are still in preclinical development with some advanced into phase I or II trials. There are several therapeutic vaccine types including: peptide vaccines, vector vaccines, recombinant protein vaccines and DNA vaccines. Most of them are focused on generating cytotoxic CD8⁺T cells in addition to antibody response.^{38,39}

Peptide vaccines - are one of the earliest vaccines, peptide based, with different adjuvants. Their main role is to induce T helper cell (Th1) and cytotoxic T cell response in chronic HCV patients.

Vector vaccines - are delivered by attenuated virus vectors. Vaccines based on modified virus of Ankara (MVA) have been shown to induce INF γ secretion by CD4⁺T and CD8⁺T. Vaccines based on adenoviral vectors were shown to induce HCV-specific T-cell response in chimpanzees.

Recombinant protein vaccines - with these types of vaccines, specific HCV response is generated by a strong Th1 adjuvant combined with recombinant HCV proteins.

DNA vaccines - are using naked DNA delivered by electroporation.³⁸⁻⁴¹

1.7 Current treatment.

Approximately 3% of the worldwide population is infected with the hepatitis C virus, with an anticipated 3-4 million individuals newly infected each year. Circa 70% of infected individuals will develop chronic hepatitis C. Cirrhosis, hepatocellular carcinoma and liver failure are the main progressive liver diseases which can develop

in untreated patients within 25-30 years. The mortality rate associated with HCV has risen to 2-5% per year. End stage liver disease due to HCV infection represents the major indication for liver transplantation in the Western world.⁴²

A protective vaccination against HCV does not exist, and standard therapy with interferon (IFN) or pegylated interferon (Peg-IFN) plus ribavirin (RBV) (**1.1**, Figure 1.3) is related to a long-term duration (12-72 weeks). Furthermore the dual regimen is not HCV-specific and relies on enhancing the host immune response.⁴³ In May 2011, the Food and Drug Administration (FDA) approved two novel protease inhibitors: telaprevir and boceprevir for the treatment of HCV. However both of these drugs still need to be co-administrated with the standard of care (SOC).⁴⁴

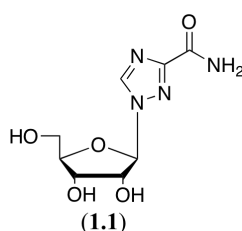


Figure 1.3. Structure of ribavirin.

Peg-IFN/RBV based therapy is often connected with side effects such as depression, anaemia, flu-like symptoms, fatigue, cognitive dysfunction, cutaneous reaction, gastrointestinal symptoms, pulmonary effects, cytopenias, thyroid dysfunction, retinopathy and others.⁴² Interferon is also administrated as subcutaneous injection, which is not preferred by the patients.⁴³

Responses to antiviral therapy of hepatitis C are grouped into three general patterns:

- Sustained virological response (SVR)
- End-of treatment response and relapse
- Non-response.

An SVR is defined as achieving undetectable levels of HCV RNA (<50 IU/ml) in serum during the treatment course and its continued absence for at least 6 months after stopping the therapy. Only 34-52% of treated individuals with HCV genotypes 1 or 4 will achieve a SVR after 48-week of therapy. By comparison approximately 80% of patients with genotypes 2 or 3 can achieve SVR during 24-weeks of treatment.⁴⁴ Furthermore virological response rates have been shown to depend also on various

host and viral factors such as: age, weight, sex, race, liver enzymes, stage of fibrosis and HCV RNA concentrations at the baseline (Figure 1.4).⁴⁵

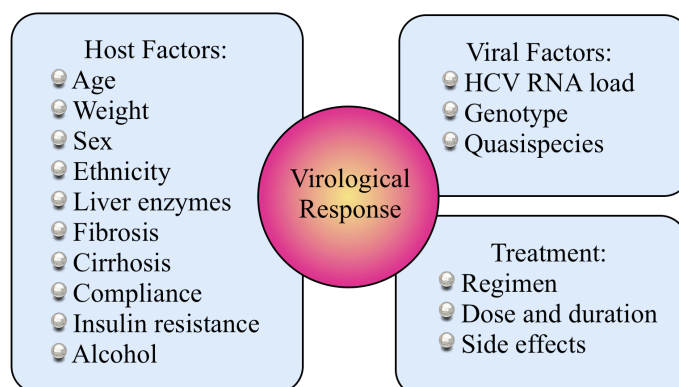


Figure 1.4. Factors associated with virological response to anti-HCV treatment.

1.7.1 Mechanism of action of therapeutic interferon.

The type-1 interferon family includes interferons- α , β , ω , and λ . All of them play a crucial role in the innate antiviral immune response. IFNs have antiviral, immunomodulatory and antiproliferative activities, but their potencies differ. IFN- α has potent antiviral activity but does not act directly on the virus or replication complex. It has been assumed that IFN treatment works by similar mechanisms to endogenous IFN, with the greater effectiveness being caused by the higher concentrations. In addition to its direct antiviral actions, IFN has important interactions with the adaptive and innate immune responses. Type-1 IFNs promote memory T-cell proliferation, prevent T-cell apoptosis and stimulate natural-killer-cell activation.^{46,47} Similarly, pegylated interferon therapy of chronic hepatitis C has been reported to be associated with enhanced T-cell responses.⁴⁸

1.7.2 Mechanism of action of ribavirin.

Ribavirin was synthesised for the first time in 1972 and was found to possess a broad spectrum of activity against broad range of RNA and DNA viruses. As a drug it was first used in mid1980s, for the treatment of respiratory syncytial virus infection in children. After the discovery of HCV, ribavirin was used as a monotherapy but the treatment did not clear HCV levels even with prolonged treatment.⁴⁹ Surprisingly, the

addition to the INF- α therapy led to marked improvement in SVR. Consequently ribavirin was approved for the treatment of HCV but only as a combination therapy with INF- α .⁵⁰

At present there are 4 proposed mechanisms of direct and indirect action of ribavirin (Figure 1.5):

- Immunomodulation
- Direct inhibition of HCV replication
- Inosine monophosphate dehydrogenase (IMPDH) inhibition
- Mutagenesis and ‘error catastrophe’^{51,52}

Immunomodulation- several studies suggest that ribavirin can enhance host T-cell-mediated immunity by altering the balance between Th1/Th2. Th1 response patterns may lead to increased activation of cytotoxic T lymphocytes (CTL) and secretion of antiviral cytokines such as interferon γ (INF γ).^{51,52}

Direct inhibition of HCV replication- Ribavirin undergoes intracellular phosphorylation by specific kinases to form the monophosphate (RMP), diphosphate (RDP) and triphosphate (RTP). RdRp incorporates RTP into the growing RNA chain opposite cytidine and uridine. This misincorporation can lead to the early chain termination and inhibition of replication. Non-phosphorylated ribavirin can also bind to the RdRp but with less affinity than RTP.^{51,52}

Inosine monophosphate dehydrogenase inhibition- IMPDH is an enzyme catalysing the *de novo* synthesis of guanosine. Inhibition of IMPDH by ribavirin can result in a depletion of the intracellular levels of guanosine triphosphate and thus inhibition of RNA synthesis. Therefore it may lead to the suppressed viral RNA replication and immunosuppression.⁵³

Mutagenesis and “error catastrophe”- As already mentioned HCV exists as many quasispecies. One feature of quasispecies is that the variety is caused by the high ongoing rate of mutations, which can occur during viral replication. It is suggested that ribavirin causes a higher frequency of mutation (mainly in the NS5A encoding region), which results in the threshold of ‘error catastrophe’. Consequently the replicative fitness of the virus is decreased and genomic diversity is narrower. As a biological result the effectiveness with which new viral particles transfect new cells is

lower, moreover ribavirin might reduce the ability of HCV to escape the immune system and thereby increase the effectiveness of antiviral therapies.⁵¹

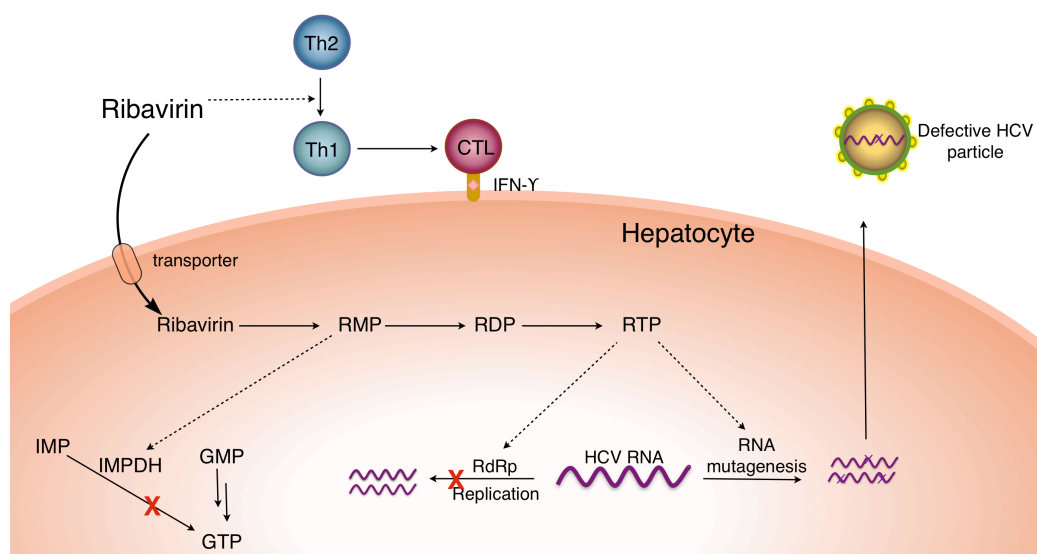


Figure 1.5. Proposed mechanism of action of ribavirin in HCV infection.

Further development of ribavirin and its analogues led to the discovery of taribavirin (1.2, Figure 1.6). As a prodrug of ribavirin, it is easily converted to ribavirin by adenosine deaminase. It possesses a better safety profile than ribavirin because of its preferential accumulation in the liver.⁵⁴ Additionally taribavirin was found to be a direct inhibitor of nucleoside phosphorylase, an enzyme responsible for the degradation of the newly formed phosphorylated species of ribavirin. First human studies of taribavirin were reported in 2004. In the phase I clinical trials taribavirin was found to be well tolerated, with rapid conversion to ribavirin and renal excretion. In phase II study, 180 chronically infected patients were randomized into 4 arms, receiving peg-INF and taribavirin or ribavirin. Patients from 3 arms receiving taribavirin were shown to develop significantly fewer cases of severe anaemia comparing to ribavirin group, however sustained viral response was lower 23-37% versus 44% of ribavirin. The results of phase II study led to two large phase III clinical trials. Studies were performed on treatment-naïve, chronically infected patients covering all genotypes. In general, SVR rates obtained for taribavirin arms were much lower than those for ribavirin group 30% vs. 58%, however anaemia rates were significantly lower in taribavirin treated patients. Although showing positive

outcomes from all phases of clinical trial, taribavirin has not yet been approved for the treatment of HCV.⁵⁵

Additional studies were performed on the L-enantiomer of ribavirin – levovirin (**1.3**, Figure 1.6). In preclinical studies for the HCV treatment, levovirin induced a Th1-signaling pathway. However levovirin does not undergo intracellular phosphorylation to its mono-, di- and triphosphate and as a result, generated virological response rates were lower than the ones for ribavirin.^{55,56} Furthermore IMPDH inhibition has not been observed. As an advantage, levovirin does not accumulate in erythrocytes and therefore no signs of haemolytic anaemia have been observed in both healthy and HCV infected patients. One randomized, double blind phase II clinical trial was conducted. Although levovirin exhibited better safety profile (less cases of anaemia), the compound did not improve rates of early virological response and therefore levovirin was not carried into further clinical development.⁵⁶

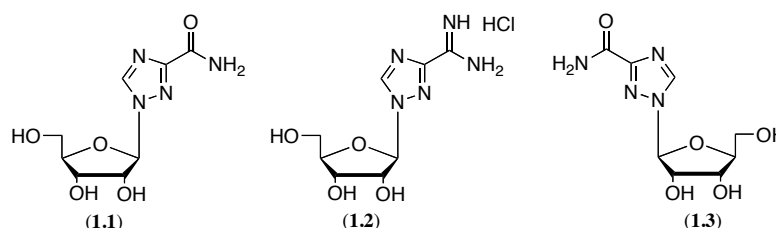


Figure 1.6. Structure of ribavirin and analogues.

1.7.3 Boceprevir and Telaprevir- Protease inhibitors

Protease inhibitors may be divided into two groups, according to the type of interaction in the active site they exploit. The first group consists of non-covalent, product based inhibitors and second one covalent reversible inhibitors. Regarding the type of structure of particular inhibitors both of these groups can be further divided into linear and macrocyclic peptidomimetics, both of which bind to the active site of the enzyme.⁵⁷ Ciluprevir (BILN 2061, Boehringer-Ingelheim) was the first specific NS3/4A macrocyclic protease inhibitor designed for the treatment of HCV. Phase I clinical trials showed a favorable pharmacokinetic profile without any serious adverse events. The compound was progressed into further development. In proof of concept two days study, ciluprevir induced 2-3 log decrease of HCV viral load, with no difference between naïve and previously treated patients. In general the drug was well

tolerated without considerable safety issues. However, further development of ciluprevir has been stopped due to cardiac toxicity observed in animals.⁵⁸

Telaprevir (**1.4**, Figure 1.7) was the second NS3-4A protease inhibitor to enter clinical trials. Telaprevir is a linear peptidomimetic that acts as a covalent inhibitor. Its structure contains α -ketoamide moiety, which act as a 'serine trap' inhibiting NS3/4A protease by binding to the enzyme active site. In phase I study, telaprevir induced a mean of 4.4 log reduction in HCV RNA levels after 14-days of treatment. In phase II, telaprevir was co-administrated with RBV and Peg-INF. All patients demonstrated undetectable levels of HCV RNA after 28-days of therapy. After successful completion of all stages of clinical trials, on 23rd of May 2011 FDA approved telaprevir as addition to the SOC. Addition of telaprevir to the RBV/Peg-INF regimen increased sustained viral response up to 75% after 48-weeks of therapy in treatment-naïve patients.^{44,57}

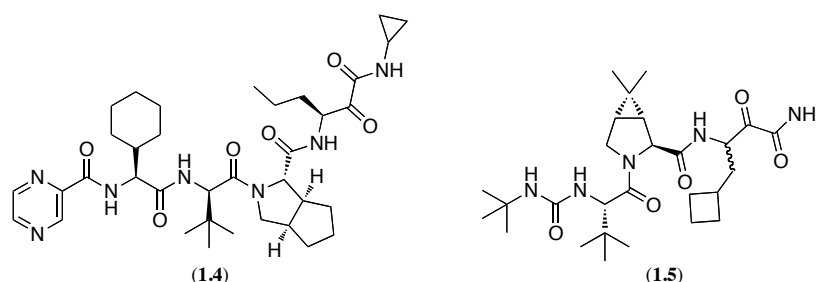


Figure 1.7. Structures of telaprevir and boceprevir.

Boceprevir (**1.5**, Figure 1.7) is another peptidomimetic inhibitor, which inhibits HCV protease. During clinical trials boceprevir was found to demonstrate dose-dependent activity in HCV-infected patients. In phase I clinical trials 4.7 log reduction in viral load was observed after 2-weeks of treatment for the highest tested dose. In subsequent study, when boceprevir was co-administrated with the SOC, mean 2.4-2.9 log reduction was seen, compared to 1.1 log for RBV/PegINF alone. All clinical trials were successfully completed and boceprevir was approved by FDA on 13th of May 2011, as a combinatorial therapy with RBV and Peg-INF. Rates of virological response for boceprevir based triple regimen were approximately 80% in treatment naïve patients.^{44,57}

A major downfall of both protease inhibitors is their inhibitory activity towards cytochrome P₄₅₀ CYP3A4/5 and many drug interactions. Additionally both

triple regimens are associated with many side effects with anemia, nausea, fatigue and dysgeusia being the most common ones.⁴⁴

Due to the fact that during clinical trials protease inhibitors have induced several cases of resistant mutations⁵⁷ it is a major interest (for both the patient care and economics) to develop new treatments that are more efficient and better tolerated by all patients. Several different approaches have been undertaken in order to address these needs. Investigators have focused their attention on the virus itself looking for specific targets associated with the viral life cycle. HCV specific drugs would include small, orally bioavailable molecules that could possibly eradicate HCV infection. The main obstacle in the development of new anti-HCV drugs is lack of readily available laboratory models of viral infection and high genetic diversity of the virus; despite this several very promising molecules have entered clinical trials and will be discussed in Chapter 2.

1.8 Polymerase inhibitors for the treatment of hepatitis C.

After revealing the structures of HCV polymerase in 1999,⁵⁹ a number of companies used structure-based drug design to facilitate the development of inhibitors to these enzymes. Currently there are 89 projects running in pre-clinical and clinical trials, including 6 projects for combinatorial therapy.⁶⁰

Polymerase inhibitors impede viral replication by binding to the NS5B polymerase. NS5B inhibitors can be classified into four major groups based on their mechanism of action. The first class consists of nucleoside and nucleotide analogue inhibitors (NI). These compounds need to be converted to the corresponding triphosphates to inhibit HCV RdRp activity. After binding to its active site, they may either act as chain terminators preventing further chain elongation or prevent the polymerase from functioning effectively. As the active site of viral polymerase exhibits highly conservative profile across all six genotypes, NIs inhibitors can exhibit high affinity towards different HCV genotypes and subtypes.⁶¹ The second group consists of non-nucleoside inhibitors (NNI) acting at several allosteric binding sites of the HCV polymerase. NNIs prevent conformational changes needed for initiation of the RNA synthesis and they do not require activation for their biological activity (unless given as prodrugs).⁶² The third class is represented by pyrophosphate analogues. They are designed to mimic the natural pyrophosphate, which is released

during the chain elongation process. Pyrophosphate analogues can bind to the active site; disabling the binding of the next incoming nucleoside triphosphate.⁶³ Additional function of pyrophosphate analogues is their ability to chelate divalent metal ions required by the RdRp.⁶⁴ The fourth class of compounds do not target HCV polymerase itself, but relays on targeting cellular proteins required for HCV polymerase function.⁶⁵

The different classes of NS5B inhibitors may act in complementary fashion and can be used for combinatorial therapy. To increase sustained virological response rates, different treatment approaches are currently under investigation. For example, individualized therapies, including duration of treatment based on the HCV RNA concentration at baseline, and trials with direct acting antiviral drugs such as inhibitors of HCV specific NS3 protease and HCV NS5B RNA-dependent RNA polymerase (RdRp).

The rationale behind the design of nucleos(t)ide inhibitors is that they can act as chain terminators. Most of nucleoside analogues can function as obligate chain terminators, due to the lack of 3'-hydroxyl group in their sugar moiety, which is required for the formation of the 3'-5' phosphodiester linkage. An incorporated 3'-modified nucleotide analogue is no longer capable of further nucleotidyl transfer reaction and as a result elongation of the viral RNA is blocked. A second class consists of non-obligate chain terminators. These nucleos(t)ide analogues possess a 3'-hydroxyl group however other modifications of the sugar moiety cause conformational changes (most likely the misalignment of the 3'-hydroxyl), sterically hindering the formation of subsequent phosphodiester bonds.^{66,67} The 2' and 4' positions of the ribose moiety have been identified as possible alteration sites, resulting in inhibition of a viral replication.⁶⁸

During the last ten years a number of ribonucleoside analogues with 2'-*O*-methyl, 2'-*C*-methyl and 4'-*C*-azido modifications have been explored for their potential to act as a specific, non-obligate chain terminators of HCV replication. These classes of compounds, although potent and selective towards viral polymerase, suffer from poor bioavailability and cell permeability, liability to metabolic degradation, and also rather poor turnover to the biologically active triphosphates.⁶⁸ Several prodrug strategies, both at the nucleoside and nucleotide levels, have been implemented to overcome these limitations and will be discussed in Chapter 2.

References:

1. Taddon RK, Yoshi YK. Recent advances in viral hepatitis: viruses and their serological markers. *Indian J Pediatr* **1980**, 47, 555-562.
2. Feinstone S, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion associated hepatitis not due to viral hepatitis type A or B. *N Engl J Med* **1964**, 292, 767- 770.
3. Seeff LB. The history of the ‘natural history’ of hepatitis C (1968-2009). *Liver Int* **2009**, 29, 89-99.
4. Berman M, Alter JH, Ishak KG, Purcell RH, Jones EA. The chronic sequelae of non- A, non- B hepatitis. *Ann Intern Med* **1979**, 91, 1-6.
5. Aach RD, Szmunes W, Mosley JW, Hollinger FB, Kahn RA, Stevens CE, Edwards VM, Werch J. Serum alanine aminotransferase of donors in relation to the risk of non- A, non- B hepatitis in recipients: the transfusion-transmitted viruses study. *N Engl J Med* **1981**, 304, 989-994.
6. Hoofnagle JH, Alter HJ. Chronic non- A, non- B hepatitis. *Prog Clin Biol Res* **1985**, 182, 63-69.
7. Alter MJ, Gerety RJ, Smallwood LA, Sampliner RE, Tabor E, Deihardt F, Frosner G, Matanoski GM. Sporadic non- A, non- B hepatitis: frequency and epidemiology in an urban U.S. population. *J Infect Dis* **1982**, 145, 886- 893.
8. Houghton M. Discovery of the hepatitis C virus. *Liver Int* **2009**, 29, 82-88.
9. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **1989**, 244, 359-362.
10. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee WS, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M. An assay for circulating antibodies to a major etiologic virus of human non- A, non- B hepatitis. *Science* **1989**, 244, 359- 362.
11. Munir S, Saleem S, Idrees M, Tariq A, Butt S, Rauff B, Hussain A, Badar S, Naudhani M, Fatima Z, Ali M, Ali L, Akram M, Aftab M, Khubaib B, Awan Z. Hepatitis C treatment: current and future perspectives. *Virol J* **2010**, 7, 296-302.

12. Chavaliez S, Pawlotsky JM. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 1.
13. Shi ST, Lai MMC. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 2.
14. Kupfer B. Hepatology. Flying Publisher **2009**, Chapter 6.
15. Polyak SJ, Klein KC, Shoji I, Miyamura T, Lingappa JR. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 2.
16. Lavie M, Goffard A, Dubuisson J. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 2.
17. Sakai A, Claire MS, Faulk K, Govinarajan S, Emerson SU, Purcell RH, Buck J. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci USA* **2003**, 100, 11646- 11651.
18. Pavlovic D, Neville DC, Argaud O, Blumberg B, Dwek RA, Fisher WB, Zitzmann N. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci USA*. **2003**, 100, 6104-6108.
19. Welbourn S, Pause A. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 5.
20. Lin C. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 6.
21. Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* **2002**, 76, 5974- 5984.
22. Shiota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S. Hepatitis C Virus (HCV) NS5A Binds RNA-dependent RNA Polymerase (RdRp) NS5B and Modulates RNA-dependent RNA Polymerase Activity. *J. Biol. Chem.* **2002**, 76, 11149- 11155.
23. He Y, Staschke KA, Tan SL. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 9.
24. Ago H, Adachi T, Yoshida A, Yamamoto M, Habuka N, Yatsunami K, Miyano M. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Cell* **1999**, 11, 1417-1426.

25. Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encirculated active site. *Nat Struct Biol* **1999**, 6, 937-943.
26. Ranjith-Kumar CT, Gutshall L, Sarisky RT, Kao CC. Multiple interactions within the hepatitis C virus RNA polymerase repress primer-dependent RNA synthesis. *J Mol Biol* **2003**, 330, 675-685.
27. Harrus D, Ahmed-El-Sayed N, Simister PC, Miller S, Triconnet M, Hagedorn CH, Mahias K, Rey FA, Astier-Gin T, Brassanelli S. Further insights into the roles of GTP and C-terminus of Hepatitis C virus polymerase in the initiation of RNA synthesis. *J Biol Chem* **2010**, 285, 32906-32918.
28. Burlone ME, Budkowska A. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. *J Gen Virol* **2009**, 90, 1055-1070.
29. Sabahi A. Hepatitis C virus entry: the early steps in the viral replication cycle. *Virol J* **2009**, 6, 117-127.
30. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. *Nature* **2005**, 933-938.
31. Ranjith-Kumar CT, Kao CC. Hepatitis C viruses. Genome and molecular biology. Norfolk (UK): Horizon Bioscience **2006**, Chapter 10.
32. www.tibotec.com/hcv_lifecycle.html
33. Sklan EH, Charuworn P, Pang PS, Glenn JS. Mechanisms of HCV survival in the host. *Nat Rev Gastroenterol Hepatol* **2009**, 6, 217-227.
34. Chisari FV. Unscrambling hepatitis C virus-host interactions. *Nature* **2005**, 436, 930-932.
35. Bode JG, Brenndorfer ED, Haussinger D. Hepatitis C virus (HCV) employs multiple strategies to subvert the host innate antiviral response. *Biol Chem* **2008**, 389, 1283-1298.
36. Myrmel H, Ulvestad E, Asjo B. The hepatitis C virus enigma. *APMIS* **2009**, 117, 427-439.
37. http://www.hcvadvocate.org/hepatitis/factsheets_pdf/genotype_FS.pdf
38. Yu CI, Chiang BL. A new insight into hepatitis C vaccine development. *J Biomed Biotech* **2010**, 2010, 1-12.
39. Halliday J, Klennerman P, Barnes. Vaccination for hepatitis C virus: closing in on an evasive target. *Expert Rev Vaccines* **2011**, 10, 659-672.

40. Castellanos M, Cinza Z, Veliz Z, Vega G, Lorenzo I, Ojeda S, Duenas-Carrera S, Alvarez-Lajonchere L, Martinez G, Ferrer E, Limonta M, Linares M, Ruiz O, Acevedo B, Torres D, Marquez G, Herrera L, Arus E. Immunization with a DNA vaccine candidate in chronic hepatitis C patients is safe, well tolerated and does not impair immune response induction after anti-hepatitis B vaccination. *J Gene Med* **2010**, 12, 107-116.
41. <http://clinicaltrials.gov>.
42. Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment- finding the right path. *Nature* **2007**, 6, 991-1000.
43. Soriano V, Peters MG, Zeuzem S. New therapies for hepatitis C virus infection. *Clin Infect Dis* **2009**, 48, 313-20.
44. www.ramsellps.com/pressroom/
45. Sarasin-Filipowicz M. Interferon therapy of hepatitis C: Molecular insights into success and failure. *Swiss Med Wkly* **2010**, 140, 3-11.
46. Tilg H. New insights into the mechanisms of interferon: an immunoregulatory and anti-inflammatory cytokine. *Gastroenterology* **1997**, 112, 1017–1021.
47. Lechner F, Wong KD, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, Robbins G, Phillips R, Klenerman P, Walker BD. Analysis of successful immune responses in persons infected with hepatitis C virus. *J Exp Med* **2000**, 191, 1499–1512.
48. Kamal SM, Ismail A, Graham CS, He Q, Rasenack JW, Peters T, Tawil AA, Fehr JJ, Khalifa Kel S, Madwar MM, Koziel MJ. PEGylated interferon alpha therapy in acute hepatitis C: relation to hepatitis C virus-specific T cell response kinetics. *Hepatology* **2004**, 39, 1721–1731.
49. Di Bisceglie AM, Conjeevaram HS, Fried MW, Sallie R, Park Y, Yudaydin C, Swain M, Kleiner DE, Mahaney K, Hoofnagle JH. Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **1995**, 123, 897-903.
50. McHutchinson JG, Poynard T. Combination therapy with interferon plus ribavirin for the treatment of chronic hepatitis C. *Semin Liver Dis* **1999**, 19, 57-65.
51. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **2005**, 436, 967-972.

52. Hofmann WP, Herrmann E, Sarrazin C, Zeuzem S. Ribavirin mode of action in chronic hepatitis C: From clinical use back to molecular mechanisms. *Liver Int* **2008**, 28, 1332-1343.
53. Lau JY, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. *Hepatology* **2002**, 35, 1002-1009.
54. Gish RG. Treating HCV with ribavirin analogues and ribavirin-like molecule. *J Antimicrob Chemother* **2006**, 57, 8–13.
55. Shields WW, Pockros PJ. Ribavirin analogs. *Clin Liver Dis* **2009**, 13, 419-427.
56. Foster GR. Past, present, and future hepatitis C treatments. *Semin Liver Dis* **2004**, 24 (Suppl 2), 97-104.
57. De Francesco R, Carfi A. Advances in the development of new therapeutic agents targeting the NS3-4A serine protease or the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *Adv Drug Deliv Rev* **2007**, 59, 1242-1262.
58. Kwong AD, McNair L, Jacobson I, George S. Recent progress in the development of selective hepatitis C virus NS3 4A protease and NS5B polymerase inhibitors. *Curr Opin Pharmacol* **2008**, 8, 522-31.
59. Ago H, Adachi T, Yoshida A, Yamamoto M, Habuka N, Yutsunami K, Miyano M. Crystal structure of RNA-dependant RNA polymerase of hepatitis C virus. *Struct Fold Des* **1999**, 7, 1417-26.
60. <http://www.hcvadvocate.org/hepatitis/hepC/HCVDrugs.html>
61. Furman PA, Lam AM, Murakami E. Nucleoside analogue inhibitors of hepatitis C viral replication: recent advances, challenges and trends. *Future Med Chem* **2009**, 1, 1429-1452.
62. Betzi S, Eydoux C, Bussetta C, Blemont M, Leyssen P, Debarnot C, Ben-Rahou M, Haiech J, Hibert M, Gueritte F, Grierson DS, Romette JL, Guillemot JC, Neyts J, Alvarez K, Morelli X, Dutartre H, Canard B. Identification of allosteric inhibitors blocking the hepatitis C virus polymerase NS5B in the RNA synthesis initiation step. *Antiviral Res* **2009**, 84, 48-59.
63. Tomei L, Altamura S, Bartholomew L, Bisbocci M, Bailey C, Bosserman M, Cellucci A, Forte E, Incitti I, Orsatti L, Koch U, De Francesco R, Olsen DB,

- Carroll SS, Migliaccio G. Characterization of inhibition of hepatitis C virus RNA replication by nonnucleosides. *J Virol* **2004**, 78, 938-946.
64. Summa V, Petrocchi A, Pace P, Matassa VG, De Francesco R, Altamura S, Tomei L, Koch U, Neuner P. Discovery of alpha, gamma-diketo acids as potent selective and reversible inhibitors of hepatitis C virus NS5B RNA-dependent RNA polymerase. *J Med Chem* **2004**, 47, 14-17.
65. He Y, Duan W, Tan SL. Emerging host cell targets for hepatitis C therapy. *Drug Discov Today* **2007**, 12, 209-217.
66. Tomei A, Altamura S, Paonessa G, De Francesco R, Migliaccio G. HCV antiviral resistance: the impact of in vitro studies on the development of antiviral agents targeting the viral NS5B polymerase. *Antivir Chem Chemother* **2005**, 16, 225-245.
67. Gluzman Y, Ostrander M. Method for treating RNA viral infections by using RNA chain terminators. US07/839,728.
68. De Clercq E. The design of drugs for HIV and HCV. *Nature Rev Drug Discov* **2006**, 6, 1001-1018.

CHAPTER TWO

2. Anti-HCV nucleos(t)ide prodrugs

2.1 Prodrugs.

The term prodrug was defined in 1958 by A. Albert and was expanded over the years.¹ Prodrugs are pharmacological substances that are administered into the body in an inactive or significantly less active form. Once administered, the prodrug undergoes chemical and/or enzymatic reaction to form an active metabolite. Chemical transformation does not depend on the presence or relative amounts of metabolizing enzymes, and therefore less interpatient variability is seen.² Prodrugs in general are most often designed to optimize 'drug-like' properties (absorption, distribution, metabolism and excretion (ADME)) and overcome barriers through a molecular approach rather than a formulation approach. Application of a prodrug technology can enhance bioavailability and permeability of a drug, increase its metabolic stability and prolong half-life. Furthermore better site-direct delivery can be achieved, while systemic exposure and unwanted tissue/organ specific toxicity can be decreased.³

There are several methods for prodrug classification, including those based on therapeutic area (e.g. antiviral prodrugs, anti-inflammatory prodrugs), site of conversion (e.g. intracellular or extracellular), mechanism of activation, or chemical properties of the promoiety.⁴ Based on the chemical nature of linkages or carrier moieties, prodrugs can be classified into 4 major categories:

Carrier-linked prodrugs are drugs that have been attached through metabolically labile linkages to another molecule also called a promoiety, which can be removed enzymatically releasing the active molecules. A promoiety is not necessary for the activation of the prodrug, but may bring an advantage of increased water or lipid solubility.⁵

There are two main types of carrier prodrugs: bipartate and tripartate. A bipartate prodrug consists of carrier and active drug, which are directly linked. A tripartate contains a spacer between the drug and carrier. Enzymatic cleavage removes the carrier, and then the spacer attached to the drug molecule undergoes spontaneous hydrolysis, releasing the drug.⁵

A slight variation of the carrier-linked prodrug approach is the mutual prodrug (co-drug) approach in which the carrier also has activity.⁶

Bioprecursor prodrugs contain no promoiety but rather rely on metabolism to introduce the functionality necessary to create an active species.⁵

Macromolecular prodrugs are molecules consisting of drug linked to the macromolecule such as polyethylene glycol.⁷

Drug-antibody conjugates are molecules where drug is covalently linked to a monoclonal antibody.⁶

2.2 Anti-HCV Nucleoside prodrugs in clinical trials.

Following the discovery of the first antiviral compound (idoxuridine) in 1959, nucleoside analogues (NA) have dominated antiviral strategies for decades.⁸ A number of nucleoside analogues have been examined for their potential to provide safe and efficacious new treatment options for hepatitis C virus infection. Several structurally different nucleoside inhibitors bearing either 4'-C-azido or 2'-C-methyl modification have provided unique profiles of anti-HCV potency and have been advanced into clinical trials. Application of simple ester prodrug strategy to these nucleoside analogues has improved their pharmacokinetic properties following oral administration.⁹

2.2.1 Valopicitabine (NM283).

Valopicitabine (NM283) was the first HCV polymerase inhibitor that has entered phase IIb clinical development. NM283 - **2.1** is an orally available 3'-O-L-valinyl ester prodrug of 2'-C-methylcytidine NM107 (**2.2**, Figure 2.1).¹⁰

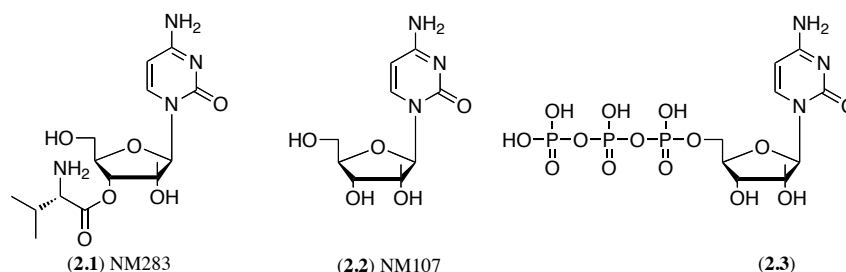


Figure 2.1. Valopicitabine - 3'-valinyl ester prodrug (NM283), 2'-C-methylcytidine nucleoside NM107 and corresponding triphosphate.

During the preliminary studies 2'-C-methylcytidine triphosphate was found to be a potent inhibitor of NS5B polymerase with $IC_{50} = 0.09\text{--}0.2\text{ }\mu\text{M}$ (**2.3**, Figure 2.1).^{11,12} When the parent nucleoside **2.2** was tested in cell-based assay it exhibited much lower activity $EC_{50} = 2\text{--}7\text{ }\mu\text{M}$. Initial pharmacokinetic studies in animal models revealed low oral bioavailability of **2.2**. To overcome this limitation and improve pharmacokinetic profile, the amino acid- 3'-O-L- valinyl ester of 2'-C-methylcytidine was prepared **2.1** and advanced into clinical trials.¹⁰ The aim of the phase I study was to evaluate safety, tolerability and pharmacokinetics of NM283 in healthy volunteers. In the phase Ib trial, after 2-week treatment (800 mg QD dose) an average of 0.7-1.00 log IU/mL reduction in circulating viral RNA levels was observed. Mild to moderate gastrointestinal adverse events were reported. A subsequent phase IIb trial was carried out on treatment-naïve genotype 1 patients and non-responders to a Peg-INF/RBV regimen. After 24-weeks a ~ 3.3 log IU/mL reduction of viraemia was demonstrated (800 mg dose). Combination treatment with Peg-INF led to a ~ 4.4 log IU/mL reduction in viral RNA after 36-weeks in treatment naïve patients while the decline in HCV genotype 1 non-responders was ca. 2 log poorer. Despite the fact that NM283 showed efficacy in clinical studies in both treatment-naïve patients and PegINF/RBV non-responders, the compound was discontinued from further development due to the significant gastrointestinal toxicity.¹³⁻¹⁵

2.2.2 Balapiravir (R1626).

4'-C-Azidocytidine (R1479, **2.4**) was discovered as a specific HCV inhibitor during the evaluation of a broad family of 4'-modified pyrimidine analogues. R1479 did not show any cytotoxicity and/or cytostatic properties at concentrations up to 2 mM.¹⁶ Its 5'-triphosphate **2.5** was shown to be potent, competitive inhibitor of HCV RdRp with $IC_{50} = 0.29\text{ }\mu\text{M}$. Due to the poor oral bioavailability of 4'-C-azidocytidine its 2',3',5'-tri-O-isobutyrate ester prodrug R1626 (**2.6**, Figure 2.2) was selected for further development.¹⁶ The introduction of isobutyrate ester groups enhanced uptake of **2.6** from the gastrointestinal tract.¹⁶ Due to the promising results of the preclinical development, this compound was progressed into clinical trials.

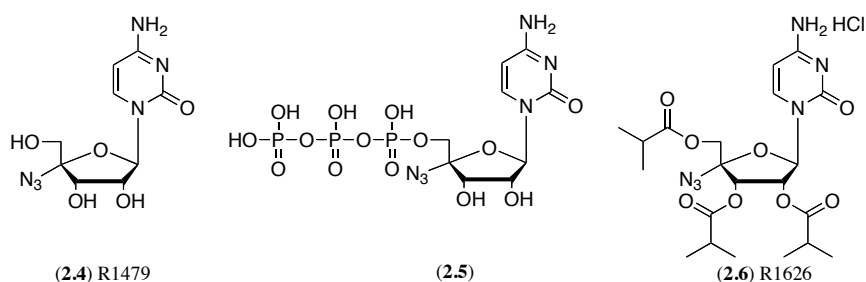


Figure 2.2. 4'-C-azidocytidine nucleoside R1479, 4'-C-azidocytidine triphosphate and nucleoside ester prodrug R1626.

During phase Ib, balapiravir demonstrated a dose and time-dependent reduction of viral load up to ~ 3.7 log IU/mL in genotype I patients, when administrated at 500-4500 mg BID. In subsequent phase IIa study, combination therapy with interferon- α and/or ribavirin gave an average of 4.2 log IU/mL decline in HCV RNA levels. At week-4 33-80% of treated patients achieved non-detectable levels of HCV RNA. Balapiravir showed high and rapid clearance of the virus, however high rates of neutropenia (39-78%) and anaemia (32%) were reported. Further development of R1626 was discontinued due to unspecified adverse events.¹⁷⁻¹⁹

2.2.3 Maricitabine (RG7128).

RG7128 (2.7) is a 3',5'-bis-isobutyryl ester prodrug of PSI-6130 (2.8, Figure 2.4).²⁰ β -D-2'-Deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130, 2.8) was found to exhibit a potent and highly specific inhibitory activity against HCV replication in cell-based assay ($EC_{50} = 1.23 \mu M$) and its triphosphate (2.9, Figure 2.3) was found to inhibit NS5B *in vitro* in low submicromolar range ($IC_{50} = 0.3-1.0 \mu M$), without any affinity towards human DNA and/or RNA polymerases.²⁰

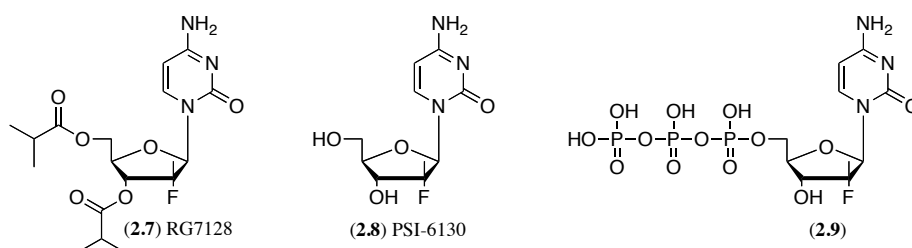


Figure 2.3. Structures of bis-isobutyryl ester prodrug RG7128, β -D-2'-Deoxy-2'-fluoro-2'-C-methylcytidine – PSI-6130 and PSI-6130 triphosphate.

PSI-6130 showed broad activity across all six HCV genotypes without any cytotoxicity in preclinical toxicology studies.²¹ PSI-6130 was advanced into clinical trials but during the human PK assessment, the compound showed only modest oral bioavailability and was found to undergo metabolic deactivation leading to the uridine analogue RO2433.²² To address these issues the bis-isobutyryl ester prodrug **2.7** of PSI-6130 was prepared (Figure 2.3). According to the published data RG7128 is currently the most advanced, simple nucleoside prodrug in clinical development.²² During phase I study, RG7128 has shown efficacy in patients infected with different HCV genotypes. Phase IIa combination trial with PegINF/RBV resulted in ~5.0 log IU/mL reduction of viraemia in genotype 1 treatment naïve subjects.²³ No serious adverse events during the treatment course were reported. RG7128 was advanced into phase IIb clinical trials and is expected to complete this study in the near future.²³

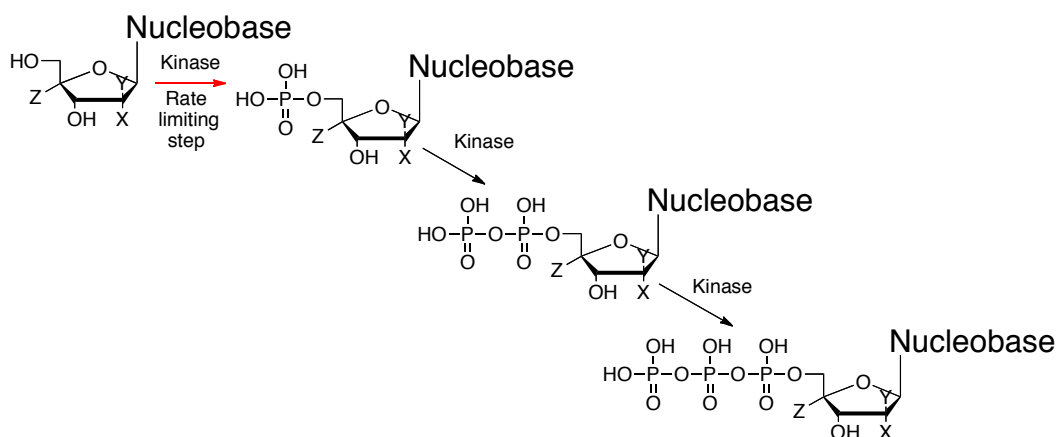
Summary of nucleoside and nucleoside prodrugs, which were evaluated in clinical trials, is reported in Table 2.1.

| Cpd | Name | Structure | Current clinical status |
|-----|-----------------------|-----------|-------------------------|
| 2.2 | NM107 | | - |
| 2.1 | Valopicitabine, NM283 | | Discontinued |
| 2.4 | R1479 | | - |
| 2.6 | Balapiravir, R1626 | | Discontinued |
| 2.8 | PSI-6130 | | - |
| 2.7 | Maricitabine, RG7128 | | Phase IIb |

Table 2.1. Summary of nucleoside and nucleoside prodrugs in the clinical trial development.

2.3 Nucleotide prodrugs - ProTides.

Although nucleoside analogues possess many advantages, they have their own challenges, which need to be overcome in order to obtain therapeutically useful agent. As already mentioned, nucleoside analogues (NA) can suffer from variable aqueous solubility and bioavailability following oral administration. Furthermore, NA activity depends upon their metabolism to form active 5'-triphosphates; therefore NAs must be good substrates for at least three different kinases. These kinases convert the nucleoside analogue to its corresponding monophosphate, then to the diphosphate and finally to the triphosphate. It is not unusual that either the parent nucleoside or its phosphates are poor substrates for one of the kinases. Quite often the first phosphorylation of the nucleoside analogue is considered as a rate-limiting step (Scheme 2.1).²⁴



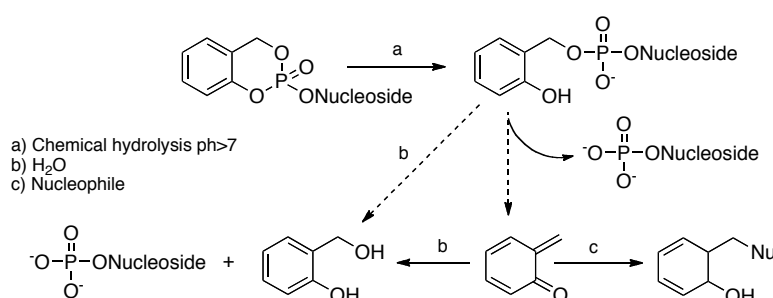
Scheme 2.1. Phosphorylation pathway of nucleoside analogues.

Additionally it is possible that the activity of kinases may decline during therapy involving long-term treatment with NAs, particularly in the case of cancer chemotherapy for example.²⁵ Administration of the pre-formed 5'-monophosphates could overcome the problem, however at physiological pH the phosphates are negatively charged and therefore too hydrophilic to efficiently cross cell membranes. Moreover 5'-nucleotidases present in the blood and cell surface can rapidly convert free nucleoside 5'-monophosphates to the corresponding nucleosides. To overcome the limitations of administering a nucleoside monophosphate, various pronucleotide approaches have been employed in order to deliver masked 5'-monophosphates and therefore improve the potential of NAs as therapeutics.²⁶ A successful

ProTide would need to possess sufficient chemical stability required for the formulation process. They should be stable in gastrointestinal fluids and undergo an efficient first-pass metabolism (for HCV activity). ProTides would have to enhance passive diffusion through the cell membrane. Once in hepatocytes, 5'-monophosphates would have to be successfully released and further phosphorylated to the active triphosphate species. Several prodrugs of modified nucleotides have demonstrated proof of concept, delivering monophosphates into cells, both *in vitro* and *in vivo*.²⁷⁻³⁰

2.3.1 Cyclosaligenyl (CycloSal) phosphotriesters.

The CycloSal approach developed by Meier,³¹ was applied to a number of nucleosides including d4T, ACV, PCV, AZT, ddA, d4A, ddI and many others.³²⁻³⁶ It does not require enzyme activation to release the monophosphate. CycloSal prodrugs were designed to generate monophosphates selectively, by a controlled, chemical hydrolysis mechanism.³² The activation mechanism is initiated by selective hydrolysis of a phenolic ester bond. Subsequently, the hydroxyl group in ortho position activates the phosphate ester at the benzyl position. Spontaneous cleavage of the diester results in the release of nucleoside 5'-monophosphate and the salicyl alcohol (Scheme 2.2). CycloSal prodrugs represent a unique mode of purely chemical activation.³²

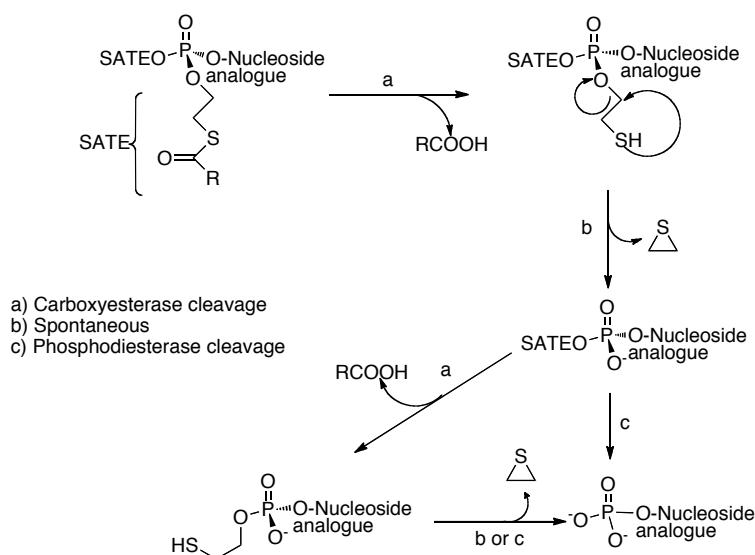


Scheme 2.2. Mechanism of activation of the first generation CycloSal ProTide.

The CycloSal approach has been further developed to the second (introduction of esterase cleavable function) and third generation.^{37,38} Recently Meier and co-workers reported delivery of nucleoside diphosphates using CycloSal technology.³⁹

2.3.2 SATE ProTides.

The SATE (S-acyl-2-thioethyl) approach was firstly reported by Imbach and Gosselin. This strategy was successfully applied to different nucleosides such as ddU, ddA, ddI, AZT, isoddA, d4T and ACV.⁴⁰⁻⁴³ The bioenzymatic activation of bis(SATE) is initiated by the carboxyesterase cleavage of the thioester moiety of the SATE groups. The generated mercaptoethylphosphotriester is unstable and undergoes intramolecular nucleophilic attack, releasing ethylene sulphide and the monoSATE phosphodiester. The resultant phosphodiester is further hydrolyzed either by a phosphodiesterase or by carboxyesterase to release the nucleoside 5'-monophosphate- this step is the same for monoSATE and cyclic SATE prodrugs (Scheme 2.3).⁴¹



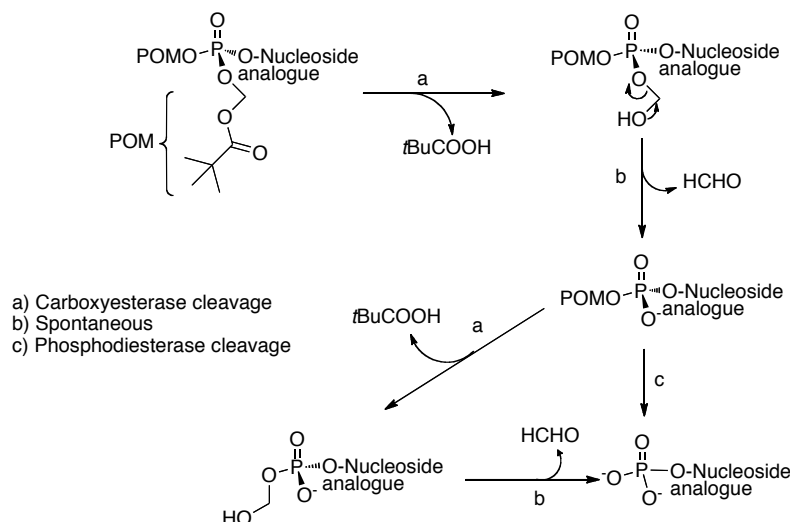
Scheme 2.3. Mechanism of activation of bis(SATE) prodrug.

One concern of this class of compounds is the release of episulfide during activation. Episulfide is a highly potent alkylating agent and known mutagen; therefore the toxicity risk associated with it has mainly limited further clinical development.⁴⁰

2.3.3 POM approach.

The pivaloxymethyl (POM) approach proposed by Farquhar and co-workers⁴⁴ was used for the development of bis(POM) triesters. This strategy was successfully applied to

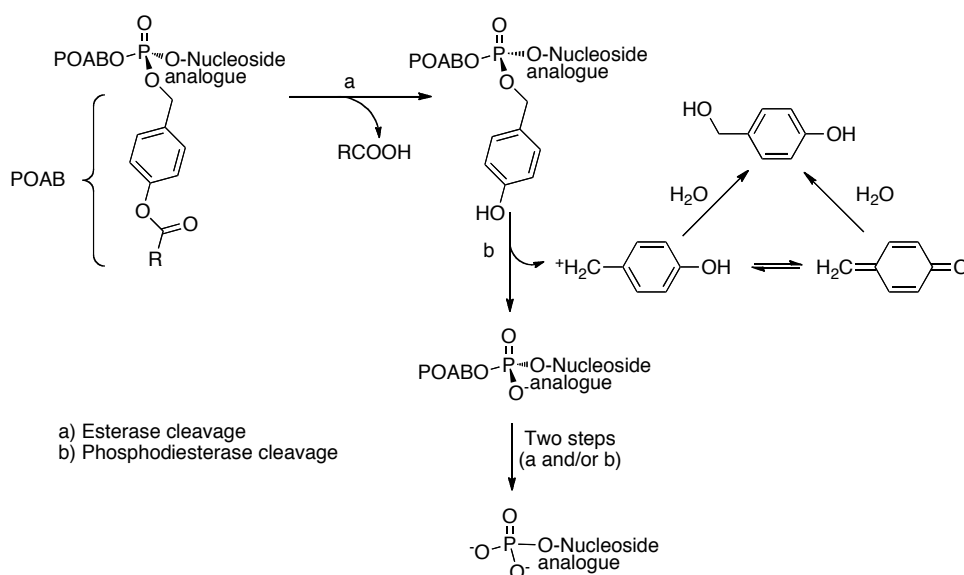
ddU, AZT and 5-fluoro-2'-deoxyuridine.^{40,41,44-46} The mechanism of activation of bis(POM) derivatives is similar to the bis(SATE) triesters. The decomposition relies on carboxyesterase-mediated hydrolysis of POM moieties, followed by intramolecular nucleophilic displacement of the hydroxyethyl group to generate mono-POM phosphodiester and formaldehyde. Release of the 5'-monophosphate is carried out by phosphodiesterase or esterase (Scheme 2.4). This step is considered as a rate-limiting step.⁴⁷



Scheme 2.4. Mechanism of activation of bis(POM) ProTide.

2.3.4 PAOB approach.

This approach was developed as an extension of the *p*-acyloxymethyl approach and was applied (as well as bis-PAOB) to the different nucleosides including AZT and ddI.⁴⁸⁻⁵⁰ The activation of PAOB requires ester hydrolysis, followed by the C-O bond cleavage and release of the phosphate. The decomposition mechanism of *para*-acyloxybenzyl phosphotriesters is analogous to the bis(POM) phosphotriesters and involves esterase-mediated hydrolysis (Scheme 2.5).⁴⁸



Scheme 2.5. Mechanism of activation of bis(PAOB) ProTide.

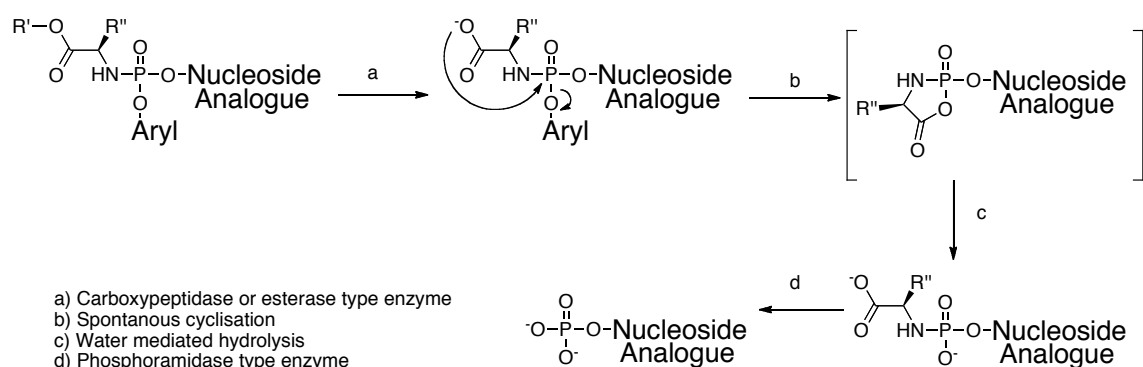
The second application of the POAB approach involved the preparation of bis(nucleosidyl) PAOB phosphotriester. *p*-Acyloxybenzyl phosphates were prepared as a homodimers and heterodimers of AZT and ddI. During the activation mechanism, one nucleoside 5'-monophosphate and one nucleoside are released.⁴⁸⁻⁵⁰

One of the hydrolysis products, the *p*-hydroxybenzyl carbonium ion, which may react with DNA, proteins or other cellular nucleophiles and this is considered as the biggest limitations of this method.⁵¹

2.3.5 Phosphoramidate di- and monoesters.

The first example of bioactive aryloxy phosphoramidates was reported by McGuigan *et al* in 1992, as prodrugs of AZT.⁵² Initially ProTides consisted of alkyl or haloalkyl phosphate triesters, subsequently one amino acid ester moiety was introduced in place of the alkyl or haloalkyl chain resulting in the alkyloxyphosphoramidates.⁵³⁻⁵⁵ Further studies led to the development of aryloxyphosphoramidate technology, which was successfully applied to a number of different nucleoside analogues, greatly improving their anticancer or antiviral activity. ProTide technology was used for the following NAs: ddA, d4A,⁵⁶ AZU,⁵⁷ AZA,⁵⁸ d4T,^{59,60} PMEa, PMPA,⁶¹ abacavir,⁶² and carbocyclic adenosine derivatives.⁶³ The most recent application of phosphoramidate technology are ProTides of *N*-acetyl glucosamine. This is the first example of ProTides used on sugars.⁶⁴

The first step of the activation of an aryloxyphosphoramidate (and subsequent release of the monophosphate species) was found to be enzyme-mediated hydrolysis of the amino acid ester moiety.^{65,66} Subsequently, the nucleophilic carboxylic acid moiety is thought to perform intramolecular attack on the phosphorus atom resulting in a putative unstable cyclic intermediate with release of the aryloxy moiety. The intermediate is then rapidly hydrolysed followed by enzyme mediated P-N bond cleavage to yield nucleoside monophosphate (Scheme 2.6).^{59,60,67,68}



Scheme 2.6. Possible mechanism of activation of aryloxy phosphoramidates.

For the description of the synthesis and structure activity relationship studies of aryloxyphosphoramidates refer to Chapter 3-5.

Wagner developed phosphoramidate monoesters as a variation of the aryloxy phosphoramidate approach. Phosphoramidate monoesters contain an amino acid ester as a masking group on one of phosphate charges. Application of only one masking group eliminates the chirality issue at phosphorus and provides better water solubility while maintaining stability in human plasma at the same time. Although cellular permeation may be more limited, this kind of prodrugs can represent an alternative method of nucleoside monophosphate delivery for intravenous applications. Activation of phosphoramidate monoesters requires phosphoramidase activity to cleave the P-N bond and proceeds directly to the monophosphate.⁶⁹

2.3.6 Nucleoside phosphorodiamidates.

Diamidate prodrugs reported by McGuigan *et al* have been proposed as potential pronucleotides since they are capable of entering cells by passive diffusion. A series of 5'-phosphorodiamidates of AZT was synthesised but the technology was not greatly developed and the synthetic pathway was not optimized.⁷⁰

Application of the diamidate strategy to the 9-[2-(phosphonomethoxy)ethoxy]adenine (PMEA) failed to produce any detectable levels of the active drug following oral administration to mice.⁷¹

Recently this approach has been successfully applied to the fructose-1,6-bisphosphatase inhibitor (**2.10**, Figure 2.4) and the agent was advanced to human clinical trials for the treatment of type 2 diabetes.⁷²

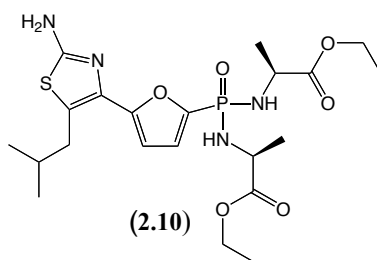


Figure 2.4. Phosphorodiamidate application to the fructose-1,6-bisphosphatase inhibitor.

Despite earlier disappointing results of the diamidate prodrug application to the different nucleoside analogues; the approach was recently successfully applied by us to the 2'-C-methylguanosine analogue.

For the description of the synthetic pathways, activation and structure activity relationship studies of phosphorodiamidate pronucleotides refer to Chapter 8.

2.3.7 Cyclic phosphoramidates.

Farquahar and co-workers reported cyclic phosphoramidates as a potential method of monophosphate delivery.⁷³ Cyclophosphamide analogues of 5-fluoro-2'-deoxyuridine and thymidine were synthesised and tested. Unlike cyclophosphamide these compounds were found to be stable to cytochrome P₄₅₀ oxidation, hydrolysis by 5'-nucleotidase, snake venom phosphodiesterase and alkaline phosphatase. As a result, no activity towards 5-fluorouracil

(5-FU) resistant cell line P-388 was found. Further studies on FUDR cyclic phosphoramidates showed that they can be metabolized to the corresponding monophosphates, resulting in lower than FUDR *in vitro* cytotoxicity against L1210 cell line.⁷³

For the synthesis and structure activity relationship studies of cyclic phosphoramidates refer to Chapter 7.

2.3.8 3',5'-Cyclic ProTides.

The design of 3',5'-cyclic ProTides was based on naturally occurring cyclic monophosphates such as cyclic adenosine monophosphate (cAMP).⁷⁴ Cyclisation of the phosphate moiety with the 3'-hydroxyl group was designed to decrease the ionic nature of the phosphate and improve lipophilicity. It was postulated that the 5'-monophosphate would be released upon the exposure to phosphodiesterases.⁷⁴ Several studies have shown that the cell uptake of different analogues of cAMP was not efficient, presumably due to the remaining charge on the phosphate centre and/or hydrolysis to the parent 5'-monophosphate prior cell membrane penetration.⁷⁵ To overcome this limitation a family of simple neutral triesters of cyclic AMP was prepared.^{74,76} A simple 3',5'-cyclic triester approach was applied to anti-HCV nucleosides giving proof of concept by delivering 5'-monophosphates into the cells both *in vitro* and *in vivo*.⁷⁷ Other cyclic approaches including 3',5'-cyclic phosphoramidates⁷⁸ and 3',5'-cyclicSATE⁷⁹ prodrugs (Figure 2.5) were also investigated.

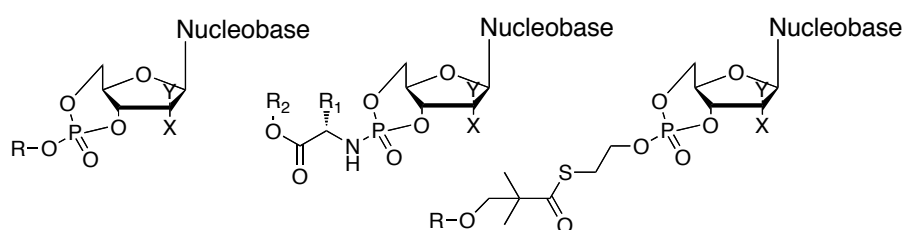


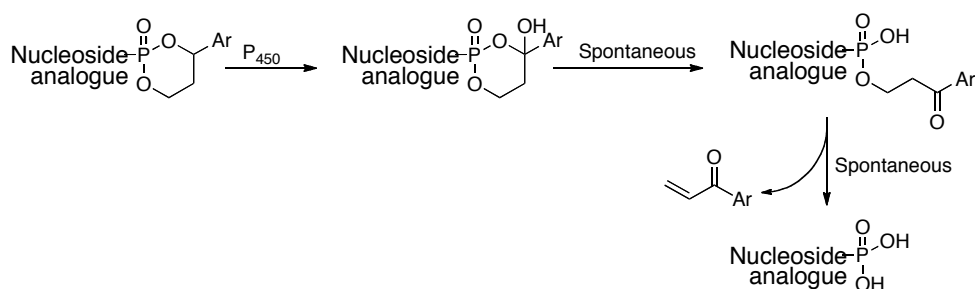
Figure 2.5. General structures of different 3',5'-cyclic ProTides.

2.3.9 HepDirect prodrugs.

In 2004 Erion *et al.* reported a specific prodrug strategy dependent on cytochrome P450 catalyzed cleavage, resulting in high liver-specific drug delivery.⁸⁰ The HepDirect

prodrugs are substituted cyclic 1,3-propanyl esters of NA phosphates or phosphonates, designed to undergo enzyme catalyzed oxidative cleavage in the liver.⁸⁰

Oxidation of the benzylic carbon by CYP3A4 results in formation of cyclic hemiketal that undergoes spontaneous ring opening and then b-elimination to give the active monophosphorylated species and aryl vinyl ketone by-product (Scheme 2.7).



Scheme 2.7. Metabolic activation of HepDirect prodrugs.

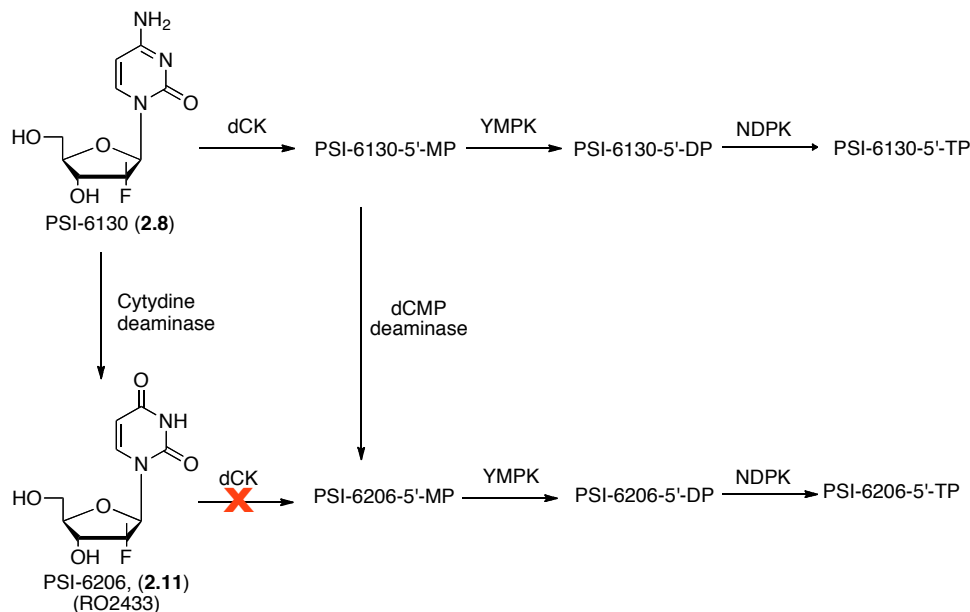
The released monophosphate is further converted to the corresponding triphosphate form.⁸⁰ The formation of aryl vinyl ketone had raised toxicological concerns, due to the possible alkylation of DNA and essential proteins. It was postulated that high levels of glutathione present in the gut and liver could detoxify vinyl ketones through a rapid and quantitative 1,4-addition reaction and thus minimize the toxicity issues.^{80,81} The HepDirect prodrug approach was successfully applied to several different nucleoside analogues resulting in the discovery of pradefovir (prodrug of adefovir). This compound was advanced into clinical trials for the treatment of hepatitis B.⁸¹

2.4 Anti-HCV nucleotide prodrugs in clinical trials.

2.4.1 PSI-7977.

As already mentioned PSI-6130 can undergo enzymatic deamination leading to the formation of poorly active uridine metabolites, eg. RO2433 (PSI-6206, **2.8**, Scheme 2.8).²² Subsequent studies indicated that the triphosphate form of RO2433 was a potent inhibitor of viral NS5B polymerase with an IC_{50} value of 0.52 μ M. Despite the potency exhibited in the enzyme assay, the nucleoside **2.11** was not active in replicon cells up to 100 μ M. Additional studies revealed that the RO2433 is a poor substrate for the deoxycytidine kinase (dCK) and

therefore the formation of RO2433- monophosphate species in human hepatocytes, was most likely accomplished by the deamination of PSI-6130-monophosphate (Scheme 2.8).^{22,82}



Scheme 2.8. Phosphorylation pathway of PSI-6130 and PSI-6206 (RO2433). 5'-MP - 5'-monophosphate, 5'-DP - 5'-diphosphate, 5'-TP - 5'-triphosphate. dCK – deoxycytidine kinase, YMPK – uridine/cytidine monophosphate kinase, NDPK – nucleoside diphosphate kinase, dCMP – deoxycytidylate deaminase.

To overcome lack of the cellular phosphorylation, the aryloxy phosphoramidate approach was applied to **2.11**, in order to deliver the monophosphate intracellularly.⁸²

SAR studies around the phosphate, resulted in the synthesis of (**2.12**, Figure 2.6) - PSI-7851 (as ca. 1:1 mixture of diastereoisomers at the phosphorus centre) exhibiting submicromolar activity in cell based assay ($EC_{90} = 0.52 \mu M$). Pharmacokinetic studies revealed that high levels of PSI-6206-5'-triphosphate in the liver can be achieved after oral administration of **2.12**, without cellular toxicities (including mitochondrial and bone marrow toxicity).⁸²

In a phase Ia trial in healthy volunteers, PSI-7851 proofed to be safe and well tolerated at all tested doses (25mg to 800mg). During multiple ascending dose study on treatment-naïve subjects, HCV RNA reduction was dose-dependant; with a maximum of 1.01 log IU/mL and 1.96 log IU/mL decline at day 3, for 200 mg and 400 mg daily dose respectively. No serious adverse and/or dose dependant effects were observed and compound was well tolerated across all tested doses.⁸³

Together with clinical development of PSI-7851, further work on separated isomers of **2.12** was performed.

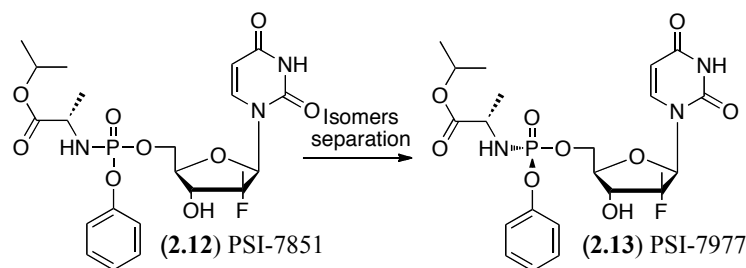


Figure 2.6. Structures of PSI-7851 (**2.12**) and PSI-7977 (**2.13**).

During the replicon clone A studies, EC_{90} values of 7.5 μM and 0.42 μM for Rp and Sp (**2.13**, Figure 2.6) isomers, respectively were reported, showing that the Sp isomer was ~ 18 times more potent than the Rp, furthermore Sp isomer was found to generate up to 14% higher concentrations of intracellular 5'-triphosphate (in human hepatocytes).^{84,85} As a result, the single Sp isomer of PSI-7851 (PSI-7977, **2.13**) was progressed into phase IIa clinical study, where HCV treatment-naïve patients were treated with 100, 200 or 400 mg QD of PSI-7977 in combination with SOC, for 28 days. Patients achieved an average of ~ 5.3 log IU/mL decline of viremia, however dose-dependent activity was not observed. There were no serious adverse events reported during the course of the treatment. All reported adverse events were mild to moderate, with joint pains, fatigue and nausea being the most common ones. Following the positive outcomes of phase IIa clinical results, a phase IIb was initiated. During the 12-week study, patients with genotype 1,2 and 3 are treated with PSI-7977 at doses of 200 mg and 400 mg QD in combination with PegINF and RBV. PSI-7977 is currently being studied in five phase II trials in patients with all HCV genotypes.⁸⁶⁻⁸⁹

2.4.2.1 IDX184.

IDX184 is a first example of a 2'-C-methylguanosine monophosphate prodrug advanced into clinical trials. IDX184 (**2.14**) represents a family of a hybrid SATE prodrugs (Figure 2.7), where two different groups: S-acetyl-2-thioethyl moiety (SATE) and benzylamine have been used to mask the pre-formed 5'-monophosphate.⁹⁰ The complete metabolic pathway of IDX184 is not yet fully disclosed, however it is postulated that the release of 5'-monophosphate relies on cytochrome 450-dependent and independent processes.⁹⁰ IDX184 was shown to be a potent inhibitor of viral replication in replicon assay $EC_{50} = 0.4 \mu\text{M}$ without any associated toxicity ($CC_{50} > 100 \mu\text{M}$).⁹¹

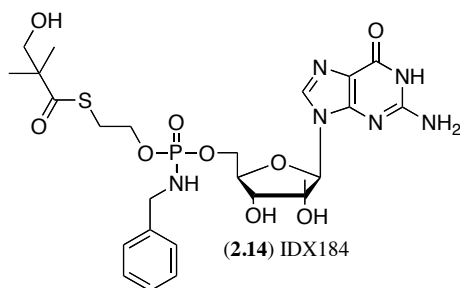


Figure 2.7. Structure of IDX184.

After successful preclinical development IDX184 was advanced into clinical trials.⁹² The aim of the phase I study was to evaluate safety, tolerability and pharmacokinetics of IDX184 in healthy volunteers. During the trial, single doses of 5-100 mg (QD, for 3 days) were generally well tolerated, without serious adverse events or dose-dependent toxicities.⁹³ The phase IIa was designed as a randomized, double blind, placebo-controlled, and dose escalation study. Treatment-naïve HCV genotype patients were treated with IDX184 in combination with PegINF/RBV for 14 days. No serious adverse events related to the tested doses of IDX184 (50 to 200 mg per day) were observed. A mean of 2.7 – 4.1 log IU/mL dose-dependent reduction of viral RNA was observed at day 14.⁹⁴ Additional phase I combination therapy with IDX320 – an NS3 protease inhibitor, was evaluated in healthy volunteers. Safety data showed three cases of liver function abnormalities during a drug-drug interaction study and the program was placed on full clinical hold, awaiting resolution of the adverse events.⁹⁵ The Food and Drug Administration (FDA) removed full clinical hold and IDX184 was placed on partial hold, after revealing that the toxicity was most likely caused by the IDX320. In July 2011, phase IIb trial of IDX184 in combination with SOC under a partial clinical hold was initiated.⁹⁶

2.4.2.2 INX-08189.

INX-08189 represents the second example of a 2'-C-methylguanosine 5'-monophosphate prodrug, also advanced into clinical trials. This compound is an aryloxy phosphoramidate double prodrug of 2'-C-methylguanosine (Figure 2.8). For the synthesis and biological evaluation refer to Chapter 5-6.

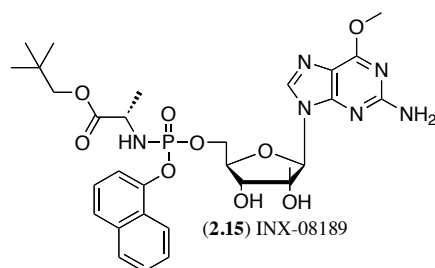


Figure 2.9. Structure of INX-08189.

2.4.2.3 PSI-938.

PSI-938 represents a 3',5'-cyclic prodrugs of 6-substituted 2'-deoxy-2'- α -fluoro-2'-*C*-methylguanosine. PSI-352938 - pure Rp isomer (**2.15**, Figure 2.9) was found to be a potent inhibitor of HCV replication in replicon assay ($EC_{90}=1.37\ \mu\text{M}$) without any cytotoxicity up to $100\ \mu\text{M}$.

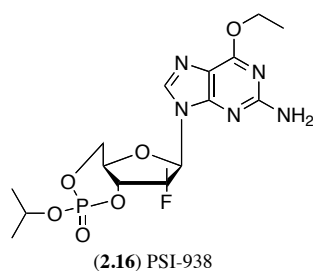


Figure 2.9. Structure of PSI-938.

Studies in animal models revealed production of high levels of 2'-deoxy-2'- α -fluoro-2'-*C*-methylguanosine triphosphate in rat liver, after oral administration of $50\ \text{mg/kg}$. The metabolic pathway of **2.15** is not yet fully disclosed.⁹⁷

In phase I clinical studies safety profile, tolerability and pharmacokinetics of PSI-352938 in healthy subjects was evaluated. The compound was generally well tolerated at all tested doses (100 to $800\ \text{mg}$). No serious adverse events or discontinuations were observed. PSI-352938 was progressed into phase Ib clinical trials. In a blind, randomized and placebo controlled study carried out on 40 treatment-naïve patients, **2.15** was administrated at doses of $100\ \text{mg}$ to $300\ \text{mg}$ QD and $100\ \text{mg}$ BID during 7-days treatment course. At day 8, a mean HCV RNA change from baseline of $3.94\ \log\ \text{IU/mL}$, $4.31\ \log\ \text{IU/mL}$, $4.64\ \log\ \text{IU/mL}$, and $4.59\ \log\ \text{IU/mL}$ in patients receiving $100\ \text{mg}$ QD, $200\ \text{mg}$ QD, $300\ \text{mg}$ QD and $100\ \text{mg}$ BID respectively was found. PSI-938 was generally well-tolerated at all tested doses. There were no reported adverse events or discontinuations over the study. Further development of the

compound as a combinatorial therapy with PSI-7977 and PSI-7977/RBV was performed. During safety monitoring, several cases of liver abnormalities were observed in patients receiving 300 mg dose of PSI-938 and compound was placed on clinical hold.^{89,98-100}

All nucleotide prodrugs (ProTides), that were/are evaluated in clinical trials are summarised in Table 2.2.

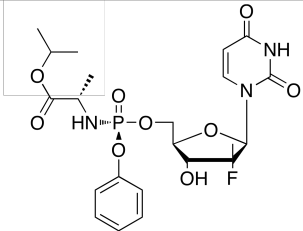
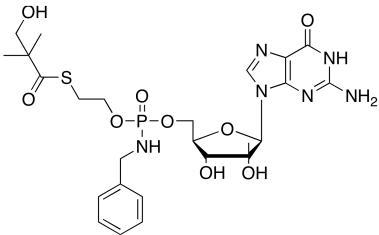
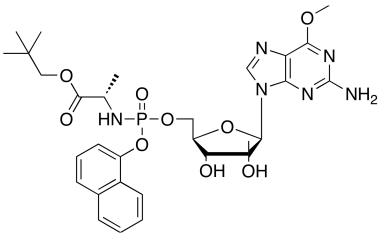
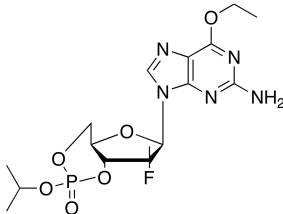
| Cpd | Name | | Current clinical status |
|------|-----------|--|---------------------------------------|
| 2.13 | PSI-7977 |  | Phase IIb |
| 2.14 | IDX184 |  | Phase IIb under partial clinical hold |
| 2.15 | INX-08189 |  | Phase IIa |
| 2.16 | PSI-938 |  | On hold |

Table 2.2. Summary of nucleotide prodrugs (ProTides) in the clinical trial development.

2.5 Aim of work.

Hepatitis C virus infection is a major health problem that leads to chronic liver disease, including cirrhosis and hepatocellular carcinoma, in a substantial number of infected individuals. Efforts to discover more effective drugs to treat HCV-infected patients have focused on nucleoside analogues with improved exposure, efficacy and selectivity.

The 2'-C- β -methylguanosine triphosphate has been known as a potent inhibitor of HCV RNA polymerase for some time, however the parent nucleoside was found to be only moderately active in the cell based assays, possibly due to the poor intracellular phosphorylation.

The following Chapters describe the synthesis of novel ProTides of 2'-C-methylguanosine and related analogues, designed to bypass the rate-limiting initial phosphorylation of the parent nucleosides and to enhance passive cellular uptake. Their biological evaluation (*in vitro* and *in vivo*) and enzymatic assays are performed to investigate a possible mode of activation.

My objectives were focused on application of ProTide technology to 2'-C- β -methylguanosine and related analogues and their biological evaluation against hepatitis C virus to generate pursuable lead and to seek clinical progression.

References:

1. Albert A. Chemical aspects of selective toxicity. *Nature* **1958**, 182, 421-22.
2. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, Savolainen J. Prodrugs: design and clinical applications. *Nature Rev Drug Des* **2008**, 7, 255-270.
3. Huttunen KM, Raunio H, Rautio J. Prodrugs-from serendipity to rational design. *Pharmacol Rev* **2011**, 63, 750-771.
4. Wu KM. A new classification of prodrugs: Regulatory perspectives. *Pharmaceuticals* **2009**, 7, 77-81.
5. Verma A, Verma B, Prajapati SK, Tripathi K. Prodrugs as a chemical delivery system: A review. *Asian J Research Chem* **2009**, 2, 100-103.
6. Stella V. J., *et al.* Prodrugs: Challenges and rewards. Part 1. **2007**, Springer
7. Bagshawe KD, Sharma SK, Springer CJ, Rogers GT. Antibody directed enzyme prodrug therapy (ADEPT). *Ann Oncol* **1994**, 5, 879-891.
8. Wagner C. R., Iyer V. V., McIntee E. J. Pronucleotides: Toward the *in vivo* delivery of antiviral and anticancer nucleotides. *Med Res Rev* **2000**, 6, 417-451
9. De Clercq E. The design of drugs for HIV and HCV. *Nature Rev Drug Discov* **2006**, 6, 1001-1018.
10. Pierra C, Amador A, Benzaria S, Cretton-Scot E, D'Amours M, Mao J, Mathieu S, Moussa A, Bridges EG, Standring DN, Sommadossi JP, Storer R, Gosselin G. Synthesis and pharmacokinetics of Valopicitabine (NM283), an efficient prodrug of the potent anti-HCV agent 2'-C-Methylcytidine. *J Med Chem* **2006**, 49, 6614-6620.
11. Le Pogam S, Jiang WR, Leveque V, Rajyaguru S, Ma H, Kang H, Jiang S, Singer M, Ali S, Klumpp K, Smith D, Symons J, Cammack N, Najera I. In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. *Virology* **2006**, 351, 349-359.
12. Ali S, Leveque V, LePogam S, Ma H, Philipp F, Inocencio N, Smith M, Alker A, Kang H, Najera I, Klumpp K, Symons J, Cammack N, Jiang WR. Selected replicon variants with low-level *in vitro* resistance to the hepatitis C virus NS5B polymerase inhibitors PSI-6130 lack cross-resistance with R1479. *Antimicrob Agents Chemother* **2008**, 52, 4356-4369.

13. Bichko V, Lalloo L, Soubasakos M, LaColla M, Tausek MM, Gillum J, Standring DN. Valopicitabine (NM 283) is fully active against known HCV protease resistance mutations in vitro. *J Hepat* **2007**, 46, (Suppl 1) S163.
14. Sherman KE, Lawitz E, O'Brien CB, Godofsky E, Brown NA. Valopicitabine (NM283) plus peg-interferon for chronic hepatitis C in treatment naïve and nonresponders patients: interim phase IIb results. *J Clin Virol* **2006**, 36, (Suppl 2) S24.
15. Kwong AD, McNair L, Jacobson I, George S. Recent progress in the development of selective hepatitis C virus NS3*4A protease and NS5B polymerase inhibitors. *Curr Opin Pharmacol* **2008**, 8, 522-531.
16. Smith DB, Martin JA, Klumpp K, Baker SJ, Blomgren PA, Devos R, Granycome C, Hang J, Hobbs CJ, Jiang WR, Laxton C, Le Pogam S, Leveque V, Ma H, Maile G, Merrett JH, Pichota A, Sarma K, Smith M, Swallow S, Symons J, Vesey D, Najera I, Cammack N. Design, synthesis, and antiviral properties of 4'-substituted ribonucleosides as inhibitors of hepatitis C virus replication: The discovery of R1479. *Bioorg Med Chem Lett* **2007**, 17, 2570-2576.
17. Roberts SK, Cooksley G, Dore GJ, Robson R, Shaw D, Berns H, Hill G, Klumpp K, Najera I, Washington C. Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. *Hepatology* **2008**, 48, 398-406.
18. Pockros PJ, Nelson D, Godofsky E, Rodriguez-Torres M, Everson GT, Fried MW, Ghalib R, Harrison S, Nyberg L, Shiffman ML, Najera I, Chan A, Hill G. R1626 plus peginterferon Alfa-2a provides potent suppression of hepatitis C virus RNA and significant antiviral synergy in combination with ribavirin. *Hepatology* **2008**, 48, 385-397.
19. http://www.roche.com/investors/ir_update/inv-update-2008-10-21.htm
20. Stuyver LJ, McBrayer TR, Tharnish PM, Clark J, Hollecker L, Lostia S, Nachman T, Grier J, Bennett MA, Xie MY, Schinazi RF, Morrey JD, Julander JL, Furman PA, Otto MJ. Inhibition of hepatitis C replicon RNA synthesis by β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine: a specific inhibitor of hepatitis C virus replication. *Antivir Chem Chemother* **2006**, 17, 79-87.

21. Murakami E, Bao H, Ramesh M, McBrayer T, Whitaker T, Sandiego-Hernandez B, Stuyver L, Schinazi RF, Obikhod A, Otto MJ, Furman PA. Mechanism of activation of β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine and Inhibition of Hepatitis C virus NS5B RNA polymerase. *Antimicrobial Agents Chemother* **2007**, 51, 503-509.
22. Ma H, Jiang WR, Robledo N, Leveque V, Ali S, Lara-Jaime T, Masjedizadeh M, Smith DB, Cammack N, Klumpp K, Symons J. Characterization of the metabolic activation of hepatitis C virus nucleoside inhibitor β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130) and identification of novel active 5'-triphosphate species. *J Biol Chem* **2007**, 282, 29812-29820.
23. <http://www.pharmasset.com/pipeline/rg7128.aspx>
24. Hecker SJ, Erion MD. Prodrugs of phosphonates and phosphates. *J Med Chem* **2008**, 51, 2328-2345.
25. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Nature-Leukemia* **2001**, 15, 875-890.
26. Mackman RL, Cihlar T. Prodrug strategies in the design of nucleoside and nucleotide antiviral therapeutics. *Annu Rep Med Chem* 39, 305-321 (2004).
27. Ariza ME. Current prodrug strategies for the delivery of nucleotides into cells. *Drug Des Rev Online* **2005**, 2, 373-387.
28. Mehellou Y, Balzarini J, McGuigan C. Aryloxyphosphoramidates triesters: a technology for delivering monophosphorylated nucleosides and sugars into cells. *ChemMedChem* **2009**, 4, 1779-1791.
29. Zemlicka J. Lipophilic phosphoramidates as antiviral pronucleotides. *Biochim Biophys Acta* **2002**, 1587, 276-286.
30. Poijarvi-Virta P. Prodrug approaches of nucleotides and oligonucleotides. *Curr Med Chem* **2006**, 13, 3441-3465.
31. Meier C. Pro-nucleotides – Recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. *Synlett* **1998**, 233-242.
32. Lorey M, Meier C, De Clercq E, Balzarini J. Cyclo-saligenyl-5-fluoro-2'-deoxyuridinemonophosphate (cycloSal-FdUMP)-a new prodrug approach for FdUMP. *Nucleosides and Nucleotides* **1997**, 16, 1307-1310.

33. Meier C, Lorey M, De Clercq E, Balzarini J. Cyclo-Saligenyl-2',3'-dideoxy-2',3'-didehydrothymidinemonophosphate (cycloSal-D4TMP)-a new pronucleotide approach. *Nucleosides and Nucleotides* **1997**, 16, 1303-1306.
34. Meier C, Habel L., Haller-Meier F., Lomp A., Herderich M., Klocking R., Meerbach A., Wutzler P. Chemistry and anti-herpes simplex virus type 1 evaluation of cycloSal-nucleotides of acyclic nucleoside analogues. *Antiviral Chem Chemother* **1998**, 9, 389-402.
35. Meier C, Knispel T, De Clercq E, Balzarini J. CycloSal-pronucleotides of 2',3'-dideoxyadenosine and 2',3'-dideoxy-2',3'-didehydroadenosine: synthesis and antiviral evaluation of a highly efficient nucleotide delivery system. *J Med Chem* **1999**, 42, 1604-1614.
36. Meier C, Knispel T, Marquez VE, Siddiqui MA, De Clercq E., Balzarini J. CycloSal-pronucleotides of 2'-fluoro-ara and 2'-fluoro-ribo-2',3'-dideoxyadenosine as a strategy to bypass a metabolic blockade. *J Med Chem* **1999**, 42, 1615-1624.
37. Meier C. Ruppel M. F. H., Vukadinovi D., Balzarini J. Second generation of cycloSal-pronucleotides with esterase-cleavable sites: the 'lock-in'- concept. *Nucleos Nucleot Nucl* **2004**, 23, 89-115.
38. Gisch N, Balzarini J, Meier C. Enzymatically activated cycloSal-d4T-monophosphates: the third generation of cycloSal-pronucleotides. *J Med Chem* **2007**, 50, 1658-1667.
39. Jessen H, Schulz T, Balzarini J, Meier C. Bioreversible protection of nucleoside diphosphates. *Angew Chem Int Ed* **2008**, 47, 922-24.
40. Gosselin G, Girardet JL, Perigaud C, Benzaria S, Lefebvre I, Schlienger N, Pompon A, Imbach JL. New insights regarding the potential of the pronucleotide approach in antiviral chemotherapy. *Acta Biochim Pol* **1996**, 43, 195-208.
41. Lefebvre I, Perigaud C, Pompon A, Aubertin AM, Girardet JL, Kirn A, Gosselin G, Imbach JL. Mononucleoside phosphotriester derivatives with S-acyl-2-thioethyl Bioreversible phosphate-protecting groups: intracellular delivery of 3'-azido-2',3'-dideoxythymidine 5'-monophosphate. *J Med Chem* **1995**, 38, 3941-3950.
42. Perigaud C, Gosselin G, Lefebvre I, Girardet JL, Benzaria S, Barber I, Imbach J.L. Rational design for cytosolic delivery of nucleoside monophosphates: SATE and DTE

- as enzyme labile transient phosphate protecting groups. *Bioorg Med Chem Lett* **1993**, 3, 2521-2526.
43. Valette G, Pompon A, Girardet JL, Cappellacci L, Franchetti P, Grifantini M, La Colla P, Loi AG, Periguad C, Gosselin G, Imbach JL. Decomposition pathways and in vitro HIV inhibitory effects of isoddA pronucleotides: toward a rational approach for intracellular delivery of nucleoside 5'-monophosphates. *J Med Chem* **1996**, 39, 1981-1990.
44. Sastry JK, Nehete PN, Khan S, Nowak BJ, Plunkett W, Arlinghaus RB, Farquhar D. Membranepermeable dideoxyuridine 5'-monophosphate analogue inhibits human immunodeficiency virus infection. *Mol Pharmacol* **1992**, 41, 441-445.
45. Farquhar D, Kuttesch NJ, Wilkerson MG, Winkler T. Synthesis and biological evaluation of neutral derivatives of 5-fluoro-2'-deoxyuridine 5'-phosphate. *J Med Chem* **1983**, 26, 1153-1158.
46. Farquhar D, Srivastava DN, Kuttesch NJ, Saunders PP. Biologically reversible phosphate-protecting groups *J Pharm Sci* **1983**, 72, 324-325.
47. Pompon A, Lefebvre I, Imbach JL, Kahn S, Farquhar D. Decomposition pathways of the mono- and bis(pivaloyloxymethyl) esters of azidothymidine 5'-monophosphate in cell culture medium: an application of the 'on-line ISRP-cleaning' HPLC technique. *Antiviral Chem Chemother* **1994**, 5, 91-98.
48. Glazier A, Kwong C, Rose J, Buckheit R, Korba B, Abou-Donia M, Smith E, Wright GE. Antiviral phosphate prodrugs. *Antiviral Res* **1992**, 17, A66, 77.
49. Routledge A, Walker I, Freeman S, Hay A, Mahmood N. Synthesis, bioactivation and anti-HIV activity of 4-acyloxybenzyl bis(nucleosid-5'-yl) phosphates. *Nucleosides and Nucleotides* **1995**, 14, 1545-1558.
50. Thomson W, Nicholls D, Irwin WJ, Al-Mushadani JS, Freeman S, Karpas A, Petrik J, Mahmood N, Hay AJ. Synthesis bioactivation and anti-HIV activity of the bis(4-acyloxybenzyl) and mono(4-acyloxybenzyl)esters of the 5'-monophosphate of AZT. *J Chem Soc Perkin Trans I* **1993**, 11, 1239-1245.
51. Mitchell AG, Nicholls D, Irwin WJ, Freeman S. Prodrugs of phosphonoformate: the effect of parasubstituents on the products, kinetics and mechanism of hydrolysis of dibenzyl methoxycarbonylphosphonate. *J Chem Soc Perkin Trans II* **1992**, 7, 1145-1150.

52. McGuigan C, Pathirana RN, Mahmood N, Devine GK, Hay AJ. Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines, which are resistant to the action of AZT. *Antivir Res* **1992**, 17, 311-321.
53. Devine KG, McGuigan C, O'Connor TJ, Nicholls SR, Kinchington D. Novel Phosphate derivatives of zidovudine as anti-HIV compounds. *AIDS* **1990**, 4, 371-372.
54. McGuigan C, Devine KG, O'Connor TJ, Galpin SA, Jeffries DJ, Kinchington D. Synthesis and evaluation of some novel phosphoramidate derivatives of 3'-azido-3'-deoxythymidine (AZT) as anti-HIV compounds. *Antivir Chem Chemother* **1990**, 1, 107-113.
55. McGuigan C, Devine K, O'Connor TJ, Kinchington D. Synthesis and anti-HIV activity of some haloalkyl phosphoramidate derivatives of 3'-azido-3'-deoxythymidine (AZT): Potent activity of the trichloroethyl methoxyalaninyl compound. *Antiviral Res* **1991**, 15, 255-263.
56. Balzarini J, Kruining J, Wedgwood O, Pannecouque C, Aquaro S, Perno CF, Naesens L, Witvrouw M, Heijntink R, De Clercq E, McGuigan C. Conversion of 2',3'-dideoxyadenosine (ddA) and 2',3'-dideoxy-2',3'-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Lett* **1997**, 410, 324-328.
57. Perrone P, Luoni G. M, Kelleher MR, Daverio F, Angell A, Mulready S, Congiatu C, Rajyaguru S, Martin JA, Leveque V, Le ogam S, Najera I, Klumpp K, Smith DB, McGuigan. Application of the Phosphoramidate ProTide Approach to 4'-Azidouridine Confers Sub-micromolar Potency versus Hepatitis C Virus on an Inactive Nucleoside. *J Med Chem* **2007**, 50, 1840-1849.
58. Perrone P, Daverio F, Valente R, Rajyaguru S, Martin JA, Leveque V, Le Pogam S, Najera I, Klumpp K, Smith DB, McGuigan C. First Example of Phosphoramidate Approach Applied to a 4'-Substituted Purine Nucleoside (4'-Azidoadenosine): Conversion of an Inactive Nucleoside to a Submicromolar Compound versus Hepatitis C Virus. *J Med Chem* **2007**, 50, 5463-5470.
59. McGuigan C, Cahard D, Sheeka HM, De Clercq E, Balzarini J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of novel intracellular metabolite. *J. Med. Chem.* **1996**, 39, 1748-1753.

60. Balzarini J, Karlsson A, Aquaro S, Perno CF, Cahard D, Naesens L, De Clercq E, McGuigan C. Mechanism of anti-HIV action of masked alaninyl D4TMP derivatives. *Proc Natl Acad Sci USA* **1996**, 93, 7295-7299.
61. Ballatore C, McGuigan C, De Clercq E, Balzarini J. Synthesis and evaluation of novel amidate prodrugs of PMEA and PMPA. *Bioorg Med Chem Lett* **2001**, 11, 1053-1056.
62. McGuigan C, Harris SH, Daluge SM, Gudmundsson KS, McLean EW, Burnette TC, Marr H, Hazen R, Condreay LD, Johnson L, De Clercq E, Balzarini J. Application of phosphoramidate pronucleotide technology to abacavir leads to a significant enhancement of antiviral potency. *J Med Chem* **2005**, 48, 3504-3515.
63. McGuigan C, Hassan-Abdallah A, Srinivasan S, Wang Y, Siddiqui A, Daluge SM, Gudmundsson KS, Zhou H, McLean EW, Peckham JP, Burnette TC, Marr H, Hazen R, Condreay LD, Johnson L, Balzarini J. Application of phosphoramidate ProTide technology significantly improves antiviral potency of carbocyclic adenosine derivatives. *J Med Chem* **2006**, 49, 7215-7226.
64. McGuigan C, Serpi M, Bibbo R, Roberts H, Hughes C, Caterson B, Gibert AT, Verson CR. Phosphate prodrugs derived from N-acetylglucosamine have enhanced chondroprotective activity in explant cultures and represent a new lead in antiosteoarthritis drug discovery. *J Med Chem* **2008**, 51, 5807-5812.
65. McGuigan C, Tsang HW, Sutton PW, De Clercq E, Balzarini J. synthesis and anti-HIV activity of some novel chain-extended phosphoramidate derivatives of d4T (stavudine): esterase hydrolysis as a rapid predictive test for antiviral potency. *Antiviral Chem Chemother* **1998**, 9, 109-115.
66. McGuigan C, Sutton PW, Cahard D, Turner K, O'Leary G, Wang Y, Gumbleton M, De Clercq E, Balzarini J. Synthesis, anti-human immunodeficiency virus activity and esterase lability of some novel carboxylic ester-modified phosphoramidate derivatives of stavudine (d4T). *Antiviral Chem Chemother* **1998**, 9, 473-479.
67. Abraham TW, Kalman TI, McIntee EJ, Wagner CR. Synthesis and biological activity of aromatic amino acid phosphoramidates of 5'-fluoro-2'-deoxyuridine and 1- β -arabinofuranosylcytosine: evidence of phosphoramidase activity. *J Med Chem* **1996**, 39, 4569-4575.

68. Kim J, Chou TF, Griegraber GW, Wagner CR. Direct measurement of nucleoside monophosphate delivery from a phosphoramidate pronucleotide by stable isotope labeling and LC-ESI MS/MS. *Mol Pharm* **2004**, 1, 102-111.
69. Iyer VV, Griesgaber GW, Radmer MR, McIntee EJ, Wagner CR. Synthesis, in vitro anti-breast cancer activity, and intracellular decomposition of amino acid methyl ester alkyl amide phosphoramidate monoesters of 3'-azido-3'-deoxythymidine (AZT). *J Med Chem* **2000**, 43, 2266-2274.
70. Jones BCNM, McGuigan C, O'Connor TJ, Jeffries DJ, Kinchington D. Synthesis and anti-HIV activity of some novel phosphorodiamidate derivatives of 3'-azido-3'-deoxythymidine (AZT). *Aniviral Chem Chemother* **1991**, 2, 35-39.
71. Serafinowska HT, Ashton RJ, Bailey S, Harnden MR, Jackson SM, Sutton D. Synthesis and in vivo evaluation of prodrugs of 9-[2-(phosphonomethoxy)ethoxy]adenine. *J Med Chem* **1995**, 38, 1372-1379.
72. Erion MD, Van Poelje PD, Dang Q, Kasibhatla SR, Potter SC, Reddy MR, Reddy KR, Jiang T, Lipscomb WN. MB06322 (CS-917): a potent and selective inhibitor of fructose 1,6-bisphosphatase for controlling gluconeogenesis in type 2 diabetes. *Proc Natl Acad Sci USA* **2005**, 102, 7970-7975.
73. Farquhar D, Kuttesch NJ, Wilkerson MG, Winkler T. Synthesis and biological evaluation of neutral derivatives of 5-fluoro-2'-deoxyuridine 5'-phosphate. *J Med Chem* **1983**, 26, 1153-1158.
74. Gohil RN, Gillen RG, Nagyvary J. Synthesis and properties of some cyclic AMP alkyl phosphotriesters. *Nucleic Acids Res* **1997**, 12, 1691-1702.
75. Meyer RB, Stone TE. 2'-O-Acyl-6-thioinosine cyclic 3',5'-phosphates as prodrugs of thioinosinic acid. *J Med Chem* **1997**, 22, 811-815.
76. Engels J, Schlaeger EJ. Synthesis, structure, and reactivity of adenosine cyclic 3',5'-phosphate benzyl triesters. *J Med Chem* **1977**, 20, 907-911.
77. Reddy PG, Bao D, Chang W, *et al.* 2'-deoxy-2'- α -fluoro-2'- β -C-methyl 3',5'-cyclic phosphate nucleotide prodrug analogs as inhibitors of HCV NS5B polymerase: discovery of PSI-352938. *Bioorg Med Chem Lett* **2010**, 24, 7376-80.
78. Meppen M, Pacini B, Bazzo R, *et al.* Cyclic phosphoramidates as prodrugs of 2'-C-methycytidine. *Eur J Med Chem* **2009**, 44, 3765-3770.

79. Gunic E, Girardet JL, Ramasamy K, Stoisavljevic-Petkoc V, Chow S, Yeh LT, Hamatake RK, Raney A, Hong Z. Cyclic monophosphate prodrugs of base-modified 2'-C-methyl ribonucleosides as potent inhibitors of hepatitis C virus RNA replication. *Bioorg Med Chem Lett* **2007**, 17, 2452-2455.
80. Erion MD, Reddy RK, Boyer SH, Matelich MC, Gomez-Galeno J, Lemus RH, Ugarkar BG, Colby TJ, Schanzer J, Van Poelje PD. Design, synthesis and characterization of a series of cytochrome P450 3A-activated prodrugs (HepDirect prodrugs) useful for targeting phosph(on)ate-based drugs to the liver. *J Am Chem Soc* **2004**, 126, 5154-5163.
81. Erion MD, van Poelje PD, Mackenna DA, Colby TJ, Montag AC, Fujitaki JM, Linemeyer DL, Bullough DA. Liver-targeted drug delivery using HepDirect prodrugs. *J Pharmacol Exp Ther* **2005**, 312, 554-560.
82. Murakami E, Niu C, Bao H, Micolochick Steuer HM, Whitaker T, Nachman T, Sofia MA, Wang P, Otto MJ, Furman PA. The mechanism of action of beta-D-2'-deoxy-2'-fluoro-2'-C-methylcytidine involves a second metabolic pathway leading to beta-D-2'-deoxy-2'-fluoro-2'-C-methyluridine 5'-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. *Antimicrobial Agents Chemother* **2008**, 52, 458-564.
83. http://www.drugs.com/clinical_trials/pharmasset-reports-positive-preliminary-antiviral-data-psi-7851-hepatitis-c-7837.html
84. Sofia MJ, Bao D, CHnag W, Du J, Nagarathnam D, Rachakonda S, Reddy PG, Ross BS, Wang P, Zhang HR, Bansal S, Espiritu C, Keilman M, Lam AM, Steuer HM, Niu C, Otto MJ, Furman PA. Discovery of β -D-2'-deoxy-2'-fluoro-2'-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. *J Med Chem* **2010**, 53, 7202-7218.
85. Murakami E, Tolstykh T, Bao H, Niu C, Steuer HM, Bao D, Chang W, Espiritu C, Bansal S, Lam AM, Otto MJ, Furman PA. Mechanism of activation of PSI-7851 and its diastereoisomer PSI-7977. *J Biol Chem* **2010**, 285, 34337-343347.
86. Lawitz E, Lalezari J, Rodriguez-Torres M, Kowdley KV, Nelson DR, DeJesus E, McHutchinson J, Cornprost MT, Mader M, Albanis E, Symonds WT, Berrey MM. High Rapid Virological Response (RVR) with PSI-7977 daily dosing plus PEG-INF/RBV in a 28-day Phase 2a Trial. *61st Annual Meeting of the American*

Association of The Study of Liver Diseases. Boston, MA, October 29-November 2, 2010.

87. http://www.pharmasset.com/pipeline/psi_7851.aspx
88. http://www.pharmasset.com/assets/1/workflow_staging/AssetManager/115.PDF
89. http://www.natap.org/2011/HCV/121611_02.htm
90. Zhou XJ, Pietropaolo K, Chen J, Khan S, Sullivan-Bolyai J, Mayers D. Safety and pharmacokinetics of IDX184, a liver-targeted nucleoside polymerase inhibitor of hepatitis C virus, in healthy subjects. *Antimicrob Chem Chemother* **2011**, 55, 76-81.
91. Cretton-Scott E, Perigud C, Peyrottes S, Licklider L, Camire M, Larsson M, La Colla M, Hildebrand E, Lallo L, Bilello J, McCarville J, Seifer M, Liuzzi M, Pierra C, Badaroux E, Gosselin G, Surleraux D, Standring DN. In vitro antiviral activity and pharmacology of IDX184, a novel and potent inhibitor of HCV replication. *J Hepatol* **2008**, Suppl.2 S220.
92. Standring D, Lanford R, Li B, Panzo RJ, Seifer M, Larsson M, Good SS, Zhou XJ. Antiviral activity of the liver targeted nucleoside HCV polymerase inhibitor IDX184 Correlates with trough serum levels of the nucleoside metabolite of HCV-infected chimpanzees. *44th Annual Meeting of the European Association for the Study of the Liver, EASL*, Copenhagen, Denmark, 22-26 April 2009.
93. Lalezari J, Asmuth D, Casiro A, Vargas A, Dubuc Patric G, Liu W, Pietropaolo K, Zhou XJ, Sullivan-Bolyai J, Mayers D. Antiviral activity, safety and pharmacokinetics of IDX184, a liver-targeted nucleotide HCV polymerase inhibitor, in patients with chronic hepatitis C. *60TH Annual Meeting of the American Assosiaction for the Study of Liver Diseases*. Boston, MA, USA, October30-November 3, 2009.
94. Lalezari J, O’Riordan W, Poordad F, Nguyen T, Dubuc Patric G, Chen J, Zhou XJ, Sullivan-Bolyai JZ, Mayers M. A Phase IIa study of IDX184 in combination with pegylated interferon (pegINF) and ribavirin (RBV) in treatment-naïve HCV genotype 1-infected subjects. *61st Annual Meeting of the American Assosiaction for the Study of Liver Diseases*. Boston, MA, USA, October29- November 2 2010.
95. http://www.hivandhepatitis.com/hep_c/news/2010/0910_2010_b.html

96. <http://phx.corporate-ir.net/External.File?item=UGFyZW50SUQ9NDM5NTA0fENoaWxkSUQ9NDYxM DMxfrFR5cGU9MQ==&t=1>
97. Reddy PG, Bao D, Chang W, Chun BK, Du J, Nagarathanam D, Rachakonda S, Ross BS, Zhang HR, Bansal S, Espritu CL, Keilman M, Lam AM, Niu C, Streuer HM, Furman PA, Otto MJ, Sofia MJ. 2'-Deoxy-2'- α -fluor-2'- β -C-methyl 3',5'-cyclic phosphate nucleotide prodrug analogs as inhibitors of HCV NS5B polymerase: Discovery of PSI-352938. *Bioorg Med Chem Lett* **2010**, 20, 7376-7380.
98. Symonds WT, Denning JM, Albanis E, Cornprost MT, Wright R, Lai A, Berrey M. Pharmacokinetics, safety and tolerability of PSI-352938, a novel nucleotide polymerase inhibitor for HCV, following single ascending oral doses in healthy subjects. *61st Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, October 29- November 2 2010.
99. http://investorshub.adfn.com/boards/read_msg.aspx?message_id=56063911#

CHAPTER THREE

Phosphoramidate approach.

3.1 Aryl amino phosphoramidates – structure activity relationship studies.

As already described in Chapter 2, aryloxy phosphoramidates were reported for the first time in early 90's¹ and since that time the approach was successfully applied to a variety of nucleoside analogues, increasing their lipophilicity, facilitating passive diffusion and enhancing their biological activity.²

The two masking groups of the phosphate unit are represented by amino acid ester and aryl moieties. Extensive elaboration performed on different arylphosphoramidates led to the optimization of three main modification sites: the amino acid, the ester and the aryl group (Figure 3.1).

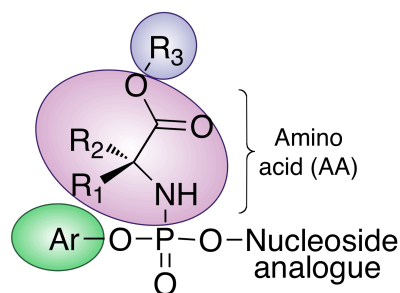


Figure 3.1. Possible modification sites in phosphoramidate moiety.

Amino Acid (AA)

Investigation of different amino acids showed that L-amino acids were found to be preferred, with L-alanine emerging as one of the most effective. Lack of substitutions at the alpha carbon (e.g. glycine) caused slight decrease of potency. However, when the alpha carbon was disubstituted, like in case of dimethylglycine, activity was retained. Bulky amino acids like L-isoleucine and L-valine proved to be less potent but exhibited improved stability in comparison with the corresponding L-alanine analogues. In general L-proline derivatives were found to be poorly active. The presence of unnatural D-amino acids resulted in loss of activity.³

Ester (R₃)

Lability of an ester moiety can dramatically change biological activity of the phosphoramidates. A range of primary, secondary, tertiary, alkyl and benzyl, linear

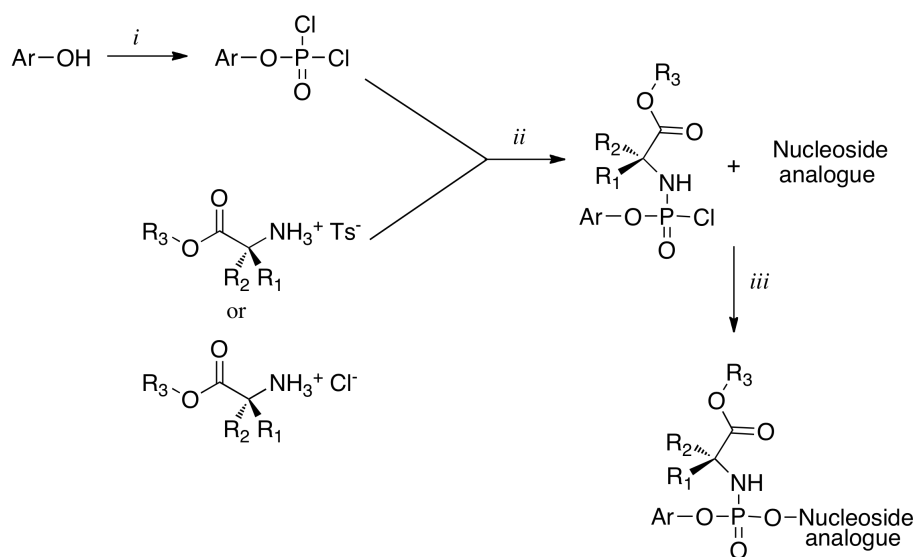
and branched esters was prepared and evaluated. Benzyl esters were found to be the most potent, whilst the activity of primary and secondary alkyl chain esters was similar. The *t*-butyl ester was found to be the least potent, probably due to its relative stability and poor esterase susceptibility.⁴

Aryl moiety (Ar)

The aryl moiety is essential as the leaving group, and may play an important role in liver targeting. The phenyl group was found to be one of the most efficient. Substitutions of the ring were acceptable, with mild electron withdrawing groups (e.g. *p*-Cl, *p*-COOMe) showing the best results.^{5,6} Recently the introduction of α -naphthol as an aryl moiety was found to increase potency of phosphoramidates comparing to the corresponding phenyl derivatives.⁷ This difference may arise from significant increases of the logP of the corresponding naphthyl phosphoramidates or their better leaving group ability.

3.2 Synthesis.

The phosphoramidate derivatives were prepared by following the synthetic procedures adapted from Van Boom *et al.*⁸ and developed within the McGuigan group.⁹ In the three-step synthesis, a nucleoside analogue is coupled with an aryl amino acid phosphorochloridate resulting in formation of the 5'-phosphoramidate (Scheme 3.1).



Reagents and Conditions: i) POCl₃, Et₃N, Et₂O, -78 °C to rt, 2h; ii) Et₃N, DCM, -78 °C to rt, 2-4h; iii) *t*BuMgCl or NMI, THF, rt, overnight.

Scheme 3.1. General synthesis of aryl phosphoramidates.

Depending on the base and nucleoside, during the reaction other regioisomers can be formed and isolated (3'-phosphoramidate and 3'5'-bis-phosphoramidate).

Each compound consists of a mixture of diastereoisomers, due to the chiral phosphate centre.

The synthesis of phosphorochloridates is discussed in more detail in the following sections.

3.2.1 Preparation of phosphorochloridates.

Phosphorochloridates were synthesised in a coupling reaction from an amino acid ester salt with an appropriate phosphorodichloridate in the presence of triethylamine (Et₃N).

3.2.1.1 Synthesis of phosphorodichloridates.

The phosphorodichloridate is the phosphorylating agent, which determines the properties of the aryl moiety in the final ProTide. Phenyl phosphorodichloridate (**3.1**) was commercially available whilst α -naphthyl (**3.1a**), β -naphthyl (**3.1b**) and *p*-NO₂-phenyl (**3.1c**) phosphorodichloridates were synthesised. In order to obtain the appropriate phosphorodichloridate, phosphorus oxychloride was added to the stirred solution of either naphthol or phenol in dry diethyl ether, in the presence of triethylamine at -78 °C (Scheme 3.1).⁹ The completion of reaction was monitored by ³¹P NMR, followed by the disappearance of POCl₃ signal. When reaction was complete the triethylamine hydrochloride salts were filtered off and the solvent was removed under reduced pressure to give clear oil used without further purifications. Table 3.1 represents the data related to the phosphorodichloridate syntheses.

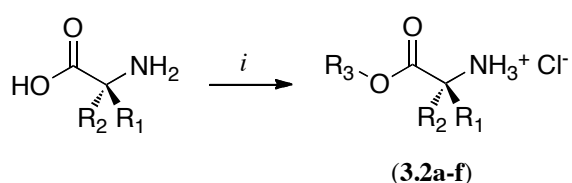
| Cpd | Aryl | Yield % | ³¹ P NMR (CDCl ₃) δ |
|-------------|-----------------------------------|---------|---|
| 3.1 | phenyl | - | 3.46 |
| 3.1a | α -naphthyl | 90 | 3.69 |
| 3.1b | β -naphthyl | 88 | 2.71 |
| 3.1c | <i>p</i> -NO ₂ -phenyl | 86 | 3.54 |

Table 3.1. Summary of synthesised phosphorodichloridates, reaction yields and ³¹P NMR shifts.

3.2.1.2 Synthesis of amino acid esters.

The synthesis of amino acid esters that were not commercially available is outlined below. There are several possible synthetic routes depending on a nature of alcohol used for the esterification reaction.

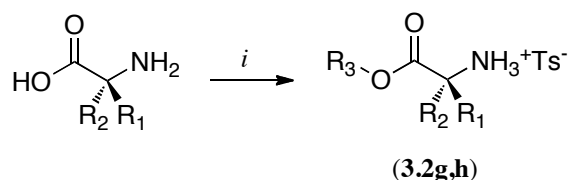
In the first method used, the amino acid and the appropriate alcohol were heated at 75 °C overnight in the presence of thionyl chloride. After crystallisation, pure amino acid esters were obtained as chloridate salts (Scheme 3.2). For the synthesised amino acid esters see Table 3.2.



Reagents and Conditions: i) Appropriate alcohol, SOCl₂, 75 °C, overnight.

Scheme 3.2. Synthesis of amino acid esters using thionyl chloride method.

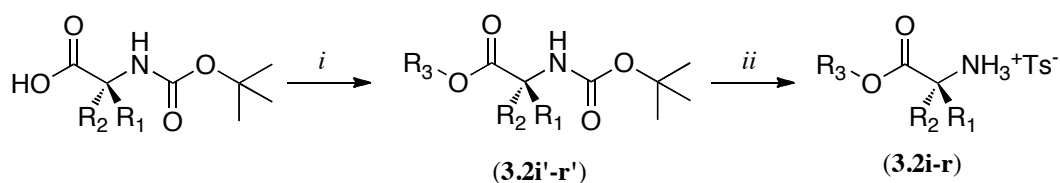
In the second method used, the amino acid and the appropriate alcohol in toluene were heated at reflux overnight in a presence of *para*-toluenesulfonic acid monohydrate (*p*TSA), in Dean-Stark apparatus. The corresponding pure amino acid esters were obtained thru crystallisation as tosylate salts (Scheme 3.3).



Reagents and Conditions: i) Appropriate alcohol, *p*TSA, toluene, reflux, overnight.

Scheme 3.3. Synthesis of amino acid esters using *p*TSA method.

The third method was used for sterically demanding substrates. In this method the Boc-protected amino acid and the appropriate alcohol were stirred overnight at room temperature in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP).¹⁰ Boc-protected amino acid esters were submitted for a deprotection reaction in ethyl acetate in the presence of *p*TSA. The pure amino acid esters were obtained as tosylate salts (Scheme 3.4).



Reagents and Conditions: i) Appropriate alcohol, DCC, DMAP, rt, overnight; ii) *p*TSA, EtOAc, 65 °C, overnight.

Scheme 3.4. Synthesis of amino acid esters using Boc-protected amino acids.

| Cpd | R ₁ | R ₂ | R ₃ (ester) | Yield % | Method used |
|------|---|-------------------------------|------------------------|---------|-------------|
| 3.2a | CH ₃ | H | <i>i</i> -propyl | 85 | 1 |
| 3.2b | CH ₃ | H | <i>n</i> -propyl | 87 | 1 |
| 3.2c | CH ₃ | H | cyclohexyl | 87 | 1 |
| 3.2d | CH ₂ CH ₂ SCH ₃ | H | <i>i</i> -propyl | 98 | 1 |
| 3.2e | H | C ₆ H ₅ | <i>n</i> -propyl | 100 | 1 |
| 3.2f | H | C ₆ H ₅ | cyclohexyl | 99 | 1 |
| 3.2g | CH ₃ | H | 2,2-dimethylpropyl | 99 | 2 |
| 3.2h | CH ₂ CH ₂ SCH ₃ | H | 2,2-dimethylpropyl | 91 | 2 |
| 3.2i | CH ₃ | H | 3,3-dimethyl-1-butyl | 59* | 3 |
| 3.2j | CH ₃ | H | 3,3-dimethyl-2-butyl | 60* | 3 |
| 3.2k | CH ₃ | H | 2-indanyl | 70* | 3 |
| 3.2l | CH ₂ CH ₂ SCH ₃ | H | cyclohexyl | 52* | 3 |
| 3.2m | CH ₂ CH ₂ SCH ₃ | H | benzyl | 67* | 3 |
| 3.2n | CH ₂ CH ₂ SCH ₃ | H | <i>S</i> -phenylethyl | 55* | 3 |
| 3.2o | CH(CH ₃)CH ₂ CH ₃ | H | cyclohexyl | 57* | 3 |
| 3.2p | CH(CH ₃)CH ₂ CH ₃ | H | 2,2-dimethylpropyl | 51* | 3 |
| 3.2r | H | C ₆ H ₅ | benzyl | 59* | 3 |

* Yield over two steps.

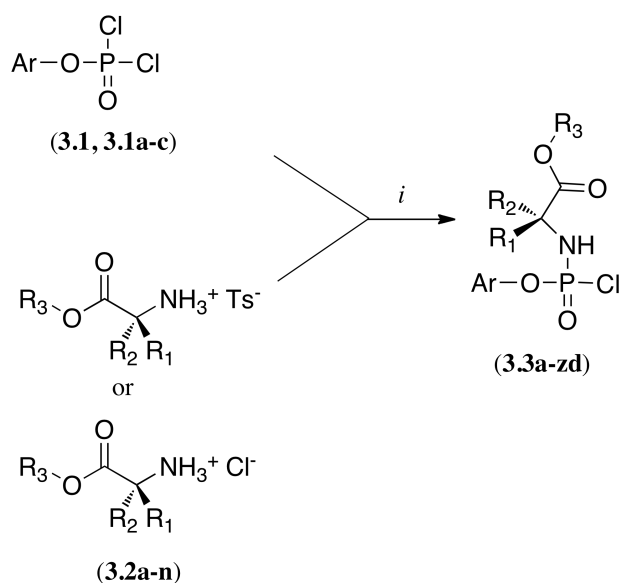
Table 3.2. Summary of amino acid esters synthesised using methods described above and isolated yields.

All esters were obtained in very good to excellent yields (Table 3.2). For 1st and 2nd method, after evaporation of reaction mixtures under vacuum, pure amino acid esters were precipitated using diethyl ether or ethyl acetate. When the 3rd method was applied, BOC-protected amino acid esters were purified by column chromatography, using a mixture of ethyl acetate/hexane as an eluent. After deprotection, which was carried out in ethyl acetate, the reaction mixture was concentrated under vacuum and kept in low temperature (0 °C). Pure amino acid ester

tosylate salts were obtained as a white precipitates. All newly synthesised amino acid esters were used for the preparation of phosphorochloridate species or phosphorodiamidate ProTides (for the synthesis of phosphorodiamidates refer to Chapter 8).

3.2.1.3 Synthesis of phosphorochloridates.

Phosphorodichloridates were coupled at low temperature (-78 °C) in the presence of Et₃N in dry DCM, with the different amino acid ester hydrochlorides or tosylate salts (Scheme 3.5). After 1h, the reaction mixture was left to reach room temperature. The formation of phosphorochloridate was monitored by ³¹P NMR, followed by the disappearance of phosphorodichloridate signal. All phosphorochloridates were purified by flash chromatography using mixtures of hexane/ethyl acetate as eluent.



Reagents and Conditions: i) Et₃N, DCM, -78 °C to rt, 2-4h.

3.2* Amino acid esters commercially available or available in the laboratory.

Scheme 3.5. Synthesis of phosphorochloridates.

Most of phosphorochloridates obtained during non-selective coupling method were represented by diastereomeric mixtures determined by the unsymmetrical substitutions on a phosphorus stereo-centre and fixed at amino acid asymmetric carbon. ³¹P NMR of synthesised compounds showed signal splitting except of the

dimethylglycine species, which were obtained as a mixture of enantiomers and therefore showing only single peak in the phosphorus spectra (Table 3.3).

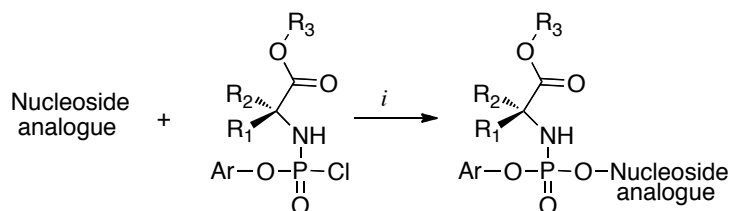
| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | Yield % | ³¹ P-NMR (CDCl ₃) δ |
|-------|-------------------------------|--|-----------------------------------|-------|------------------------|---------|--|
| 3.3a | α-naph | CH ₃ | H | L-Ala | methyl | 52 | 8.19; 7.92 |
| 3.3b | α-naph | CH ₃ | H | L-Ala | <i>i</i> -propyl | 30 | 8.29; 7.97 |
| 3.3c | α-naph | CH ₃ | H | L-Ala | <i>n</i> -propyl | 82 | 8.29, 8.04 |
| 3.3d | α-naph | CH ₃ | H | L-Ala | <i>t</i> -butyl | 69 | 8.43, 8.11 |
| 3.3e | α-naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 99 | 8.21; 7.90 |
| 3.3f | α-naph | CH ₃ | H | L-Ala | 3,3-dimethyl-1-butyl | 91 | 8.46; 8.02 |
| 3.3g | α-naph | CH ₃ | H | L-Ala | 3,3-dimethyl-2-butyl | 96 | 8.30; 8.24; 7.98; 7.95* |
| 3.3h | α-naph | CH ₃ | H | L-Ala | cyclopentyl | 65 | 8.38; 8.13 |
| 3.3i | α-naph | CH ₃ | H | L-Ala | cyclohexyl | 92 | 8.29; 7.92 |
| 3.3j | α-naph | CH ₃ | H | L-Ala | tetrahydropyranyl | 70 | 8.19; 8.00 |
| 3.3k | α-naph | CH ₃ | H | L-Ala | benzyl | 85 | 8.16; 7.92 |
| 3.3l | α-naph | CH ₃ | H | L-Ala | <i>S</i> -phenylethyl | 68 | 8.22; 7.94 |
| 3.3m | α-naph | CH ₃ | H | L-Ala | 2-indanyl | 65 | 8.16; 7.90 |
| 3.3n | Ph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 95 | 8.02; 7.73 |
| 3.3o | <i>p</i> -NO ₂ -Ph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 83 | 8.10 |
| 3.3p | α-naph | H | H | Gly | benzyl | 95 | 5.86** |
| 3.3r | α-naph | CH(CH ₃) ₂ | H | L-Val | methyl | 62 | 10.02; 9.56 |
| 3.3s | α-naph | CH(CH ₃) ₂ | H | L-Val | 2,2-dimethylpropyl | 76 | 9.84; 9.34 |
| 3.3t | α-naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 90 | 8.26; 8.05 |
| 3.3u | β-naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 99 | 9.71; 9.18 |
| 3.3v | α-naph | H | CH(CH ₃) ₂ | D-Val | benzyl | 99 | 9.71; 9.30 |
| 3.3w | α-naph | CH(CH ₃) ₂ | H | L-Val | <i>p</i> -Cl-benzyl | 99 | 9.72; 9.24 |
| 3.3x | α-naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | <i>i</i> -propyl | 67 | 8.88; 8.80 |
| 3.3y | α-naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | 2,2-dimethylpropyl | 41 | 8.92; 8.74 |
| 3.3z | α-naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | benzyl | 74 | 8.76; 8.63 |
| 3.3za | α-naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | <i>S</i> -phenylethyl | 79 | 8.78; 8.64 |
| 3.3zb | α-naph | H | C ₆ H ₁₀ | D-PhG | <i>n</i> -propyl | 59 | 7.72; 7.52 |
| 3.3zc | α-naph | H | C ₆ H ₁₀ | D-PhG | cyclohexyl | 60 | 7.82; 7.64 |
| 3.3zd | α-naph | H | C ₆ H ₁₀ | D-PhG | benzyl | 72 | 7.60; 7.42 |

AA-Amino acid; * 4 diastereoisomers present, due to the additional chiral centre of the 3,3-dimethyl-2-butyl ester (R,S); **No diastereomeric splitting as compound is a mixture of enantiomers.

Table 3.3. Yields % and ³¹P NMR shifts of phosphorochloridates with different amino acids, esters and aryl moieties.

3.2.2 Synthesis of phosphoramidates.

The phosphoramidates were obtained by the coupling of nucleoside analogues with appropriate phosphorochloridate in the presence of a base: *t*BuMgCl or NMI in room temperature in dry THF (Scheme 3.6).



Reagents and Conditions: i) *t*BuMgCl, THF, rt, overnight; or NMI, THF, rt, overnight.

Scheme 3.6. General synthesis of phosphoramidates.

It was reported by Uchiyama,¹¹ that *tert*-butylmagnesium chloride (the Grignard reagent, *t*BuMgCl) acts as a specific activator for O-selective phosphorylation. The Grignard reagent does not show selectivity towards primary hydroxyl groups (5' position), therefore using nucleoside analogues bearing more than one hydroxyl group may lead to the undesired phosphoramidate isomers like 3'-phosphoramidate as well as 3', 5'-bisphosphoramidate. Despite the lack of selectivity for primary alcohols, *tert*-butyl-magnesium chloride mediated couplings were reasonable yielding, crude mixtures were easy to purify and recovery of parent nucleoside was possible.

An alternative N-methylimidazole (NMI) synthetic procedure favours a selective phosphorylation of the primary hydroxyl group in 5'-position due to the different mechanism of action.¹² However the crude mixtures obtained from the coupling required mild acidic extraction to remove NMI and were difficult to purify. Despite its selectivity to the primary hydroxyl groups, the NMI method was not the method of first choice mainly due to the low coupling yields and problematic purifications.

For the synthesis of aryloxyphosphoramidates of 2'-C- β -methylguanosine see Chapter 4.

References:

1. McGuigan C, Pathirana RN, Mahmood N, Devine GK, Hay AJ. Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines which are resistant to the action of AZT. *Antivir Res* **1992**, 17, 311-321.
2. Mehellou Y, Balzarini J, McGuigan C. Aryloxyphosphoramidates triesters: a technology for delivering monophosphorylated nucleosides and sugars into cells. *ChemMedChem* **2009**, 4, 1779-1791.
3. McGuigan C, Tsang HW, Cahard D, Turner K, Velazquez S, Salgado A, Bidois L, Naesens L, De Clercq E, Balzarini J. Phosphoramidate derivatives of d4T as inhibitors of HIV: the effect of amino acid variation. *Antiviral Res* **1997**, 35, 195-204.
4. Knaggs MH, McGuigan C, Harris SA, Heshmati P, Cahard D, Gilbert IH, Balzarini J. A QSAR study investigating the effect of L-alanine ester variation on the anti-HIV activity of some phosphoramidate derivative of d4T. *Bioorg Med Chem Lett* **2000**, 10, 2075-2078.
5. Siddiqui AQ, Ballatore C, McGuigan C, De Clercq E, Balzarini J. The presence of substituents on the aryl moiety of the aryl phosphoramidate derivative of d4T enhances anti-HIV efficacy in cell culture: a structure-activity relationship. *J Med Chem* **1999**, 42, 393-399.
6. Siddiqui AQ, Ballatore C, McGuigan C, Zuccotto F, Gilbert IH, De Clercq E. Design and synthesis of lipophilic phosphoramidate d4T-MP prodrugs expressing high potency against HIV in cell culture: structural determinants for in vitro activity and QSAR. *J Med Chem* **1999**, 42, 4122-4128.
7. Congiatiu C, McGuigan C, Jiang WG, Davies G, Mason MD. Naphthyl phosphoramidate derivatives of BVDU as potential anticancer agents: design, synthesis and biological evaluation. *Nucleos Nucleot Nucl* **2005**, 24, 485-489.
8. Van Boom JH, Burgers PM, Crea R, Luyten WCM, Vink ABJ, Reese CB. Phosphorylation of nucleoside derivatives with aryl phosphoramidochloridates. *Tetrahedron* **1975**, 31, 2953-2959.
9. Curley D, McGuigan C, Devine KG, O'Connor TJ, Jeffries DJ, Kinchington D. Synthesis and anti-HIV evaluation of some phosphoramidate derivatives of AZT; studies on the effect of chain elongation on biological activity. *Antiviral Res* **1990**, 14, 245-256.

10. Neises B, Steglich W. Simple method for the esterification of the carboxylic acids. *Angew Chem Int Edn* **1975**, 17, 522-524.
11. Uchiyama M, Aso Y, Noyori R, Hayakawa Y. O-selective phosphorylation of nucleosides without N-protection. *J Org Chem* **1993**, 58, 373-379.
12. Farrow SN, Jones AS, Kumar A, Walker RT, Balzarini J, De Clercq E. Synthesis and biological properties of novel phosphotriesters: a new approach to the introduction of biologically active nucleotides into cells. *J Med Chem* **1990**, 33, 1400-1406.

CHAPTER FOUR

Aryloxyphosphoramidates of 2'-C- β -methylguanosine.

4.1 2'-C- β -methylpurines.

2'-C-methylpurines have been reported for the first time in late 60's¹ and since that time a number of 2'-C-branched nucleosides have been described as promising anticancer²⁻⁴ and antiviral agents.⁵ These nucleosides are usually recognised by different cellular kinasess to form their corresponding 5'-triphosphates – the active species.²⁻⁴

Several examples of 2'-modified nucleoside analogues have been reported with potential anti-HCV activity.⁶⁻⁸ RdRp is able to distinguish ribonucleoside triphosphate from 2'-deoxynucleoside triphosphates making it likely that the modification of 2'-position will be recognized and lead to chain termination.⁶

We have focus our attention on one of the 2'-modified nucleoside analogues - 2'-C- β -methylguanosine (2'CMeG) that has showed good *in vitro* activity (IC₅₀= 0.13 μ M of NTP, EC₅₀= 3.5 μ M). Most importantly, the detected level of the corresponding 5'-triphosphate *in vitro* was rather poor (0.2 intercellular NTP/pmol per 10⁶ cell). This might suggest that 2'-C- β -methylguanosine is a poor substrate for kinases responsible for conversion to its active triphosphate form.^{7,8} We decided to apply the aryloxyphosphoramidate approach to this nucleoside in order to explore the possibility of further increasing its anti-HCV activity.

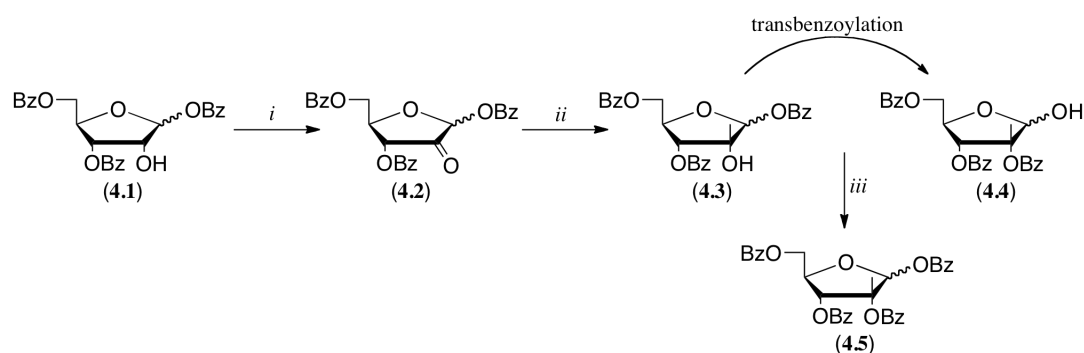
4.2 Synthesis of 2'-C- β -methylguanosine.

The first procedure for the synthesis of 2'-C-methylguanosine was reported in 1968. The nucleoside was synthesised via the reaction of 2,3,5-tri-*O*-benzoyl-2-C-methyl- β -D-ribofuranosyl chloride with chloromercuripurine.¹ However the synthetic route towards the appropriate sugar derivative required 8 steps and overall yield was low.⁹ Furthermore the glycosylation step was low yielding and regioselectivity (N7 vs. N9) was not disclosed.^{1,10} Later on several research groups reported more efficient routes using either linear or convergent approaches.^{10,11} In this work a convergent

method was used, as it is more flexible since a variety of nucleobases can be coupled to the modified sugar moiety. The key glycosylating agent - 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose was synthesised using two different routes. In the first one, the 2-position of the sugar moiety was stereoselectively alkylated to afford the desired 2-alkylsugar as a mixture of α and β anomers.¹¹ In the second method alkylated lactone was reduced to yield the desired sugar derivative as an anomeric mixture.¹²

4.2.1 Synthesis of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose (First method).

The oxidation of commercially available 1,3,5-tri-*O*-benzoyl-D-ribofuranose **4.1** with Dess-Martin periodinane in anhydrous conditions overnight at ambient temperature gave the corresponding lactone **4.2** in 95% yield. The stereoselective addition of a methyl in the β -2-position was performed in the presence of TiCl_4 and MeMgBr in diethyl ether at -78°C . The presence of the titanium reagent provided selectivity towards the ketone functionality in the presence of esters. Reaction gave an anomeric mixture of **4.3** and **4.4**. The formation of **4.4** was due to the trans benzoylation. After purification by column chromatography, a mixture of compounds **4.3** and **4.4** was used for the next step, where the remaining free hydroxyl groups were protected by 4-(dimethylamino)pyridine (DMAP) catalysed benzoylation in the presence of triethylamine (Et_3N) (Scheme 4.1).¹¹



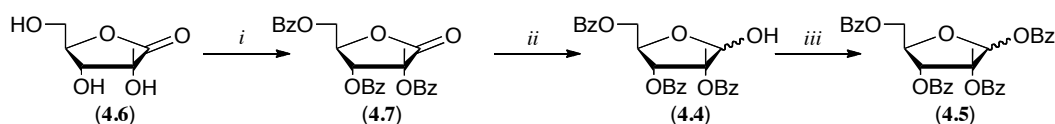
Reagents and Conditions: i) Dess-Martin periodinane, DCM, 0°C to rt, 12 h; ii) TiCl_4 , MeMgBr , THF, -78°C to -10°C , 4 h; iii) BzCl , DMAP, Et_3N , Et_2O , -10°C to rt, 3 h.

Scheme 4.1. Synthesis of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose via Dess-Martin oxidation method.

The resulting 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose **4.5** was submitted for glycosylation reaction.

4.2.2 Synthesis of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose (Second method).

The second route involved reduction of commercially available 2-*C*-methyl-D-ribo-1,4-lactone **4.6**. Firstly, ribonolactone was protected with benzoyl chloride to give compound **4.7**, which was submitted for the reduction reaction without any purification. Red-Al mediated reduction gave anomeric mixture **4.4**, which after extraction was used directly for the subsequent step. Protection with benzoyl chloride in the presence of DMAP and Et₃N gave compound **4.5** (Scheme 3.2).¹²



Reagents and Conditions: i) BzCl, DMAP, Et₃N, DME, rt, 2 h; ii) Red-Al, EtOH, -5 °C, 40 min; iii) BzCl, DMAP, Et₃N, THF, 5 °C to rt, overnight.

Scheme 4.2. Synthesis of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose via Red-Al reduction method.

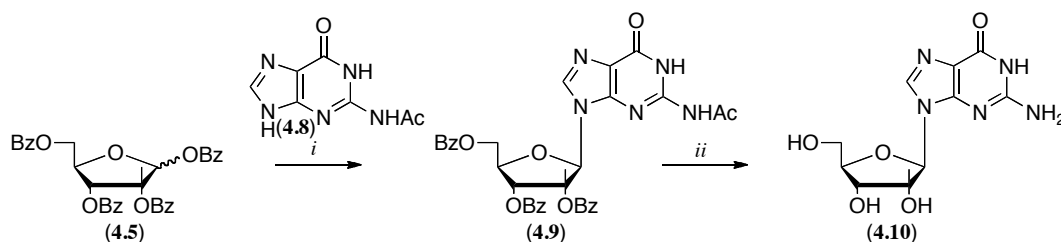
Both synthetic pathways consist of three steps. However, Red-Al method was easier to handle in terms of the reagents and reaction conditions, neither of the steps required purification. Taking in consideration all aspects of these two syntheses the Red-Al reduction method was chosen for the scale-up synthesis.

4.2.3 Coupling reaction.

Vorbrüggen *et al.* described for the first time glycosylation of persilylated *N*²-acetylguanine, under thermodynamic conditions (reflux in 1,2-dichloroethane, trimethylsilyl triflate (TMS-triflate) as a catalyst).¹³ Reaction was high yielding (79%) and gave 6:1 ratio of N9 and N7 products. In this work improved procedure of Li and Piccirilli was used.¹⁰ The coupling reaction of protected sugar derivative **4.5** and *N*²-acetylguanine **4.8** was performed in two steps: firstly, the silylated *N*²-acetylguanine was synthesised followed by the coupling reaction in the presence of TMS-triflate, using *p*-xylene as a solvent.¹⁰ Use of nonpolar *p*-xylene in high

temperature afforded formation of the thermodynamic N9-product **4.9** in 73% yield. Formation of N9 alpha anomer and/or N7 products was not observed. *N*²-acetyl-2',3',5'-tri-*O*-benzoyl-2'-*C*-β-methylguanosine was isolated by crystallisation from the mixture of chloroform and methanol.

The final step was the cleavage of protecting groups of the sugar and base through ammonolysis leading to 2'-*C*-β-methylguanosine **4.10** (Scheme 4.3).¹⁰



Reagents and Conditions: i) a) 1,1,1,3,3,3-hexamethyldisilazane, dry pyridine, reflux, 2-5 h, b) TMS-triflate, *p*-xylene, 140 °C, 6 h; ii) NH₃/MeOH, rt, overnight.

Scheme 4.3. Synthesis of 2'-*C*-β-methylguanosine.

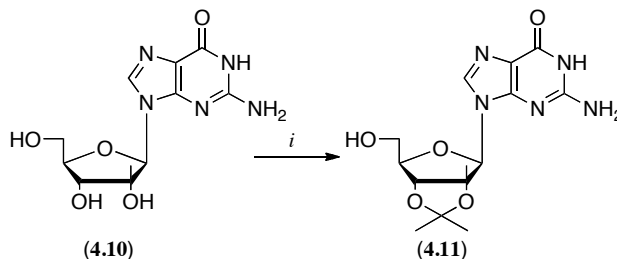
2'-*C*-β-methylguanosine **4.10** was obtained in 5 steps, with overall yield of 50%. In order to easily set up reactions and purifications during the synthetic pathway, 2'CMeG was synthesised several times on 5.0 g scale.

4.3 Synthesis of 2'3'-isopropylidene-2'-*C*-β-methylguanosine.

Several ProTide coupling attempts were performed on unprotected nucleosides resulting in either no product formation or very low coupling yields. 2'-*C*-methylguanosine was not soluble in the solvent of first choice- anhydrous tetrahydrofuran. Addition of pyridine to the reaction mixture did not resolve the solubility problems and did not result in the product formation. Reaction carried out in pyridine as a sole solvent, gave the desired product, but reaction mixture was very difficult to purify and very low yielding, therefore not suitable for scale-up synthesis.

To overcome solubility problems and to enhance 5'-phosphorylation, various protecting groups for the 2',3'-diol were investigated. The nature of the protecting group was strongly related to the stability of phosphoramidate unit. The 5'-ProTide moiety is considered as chemically fragile, however several studies confirmed their acid-stable nature.¹⁴ These findings led us to acid-labile isopropylidene protecting group which was used for the final phosphoramidate coupling.¹⁵

The synthesis in dry acetone with a catalytic amount of perchloric acid at ambient temperature overnight gave the desired protected diol **4.11** (Scheme 4.4) in 90% yield. The synthesised protected nucleoside was soluble in dry THF and reacted easily with phosphorochloridates under coupling conditions.

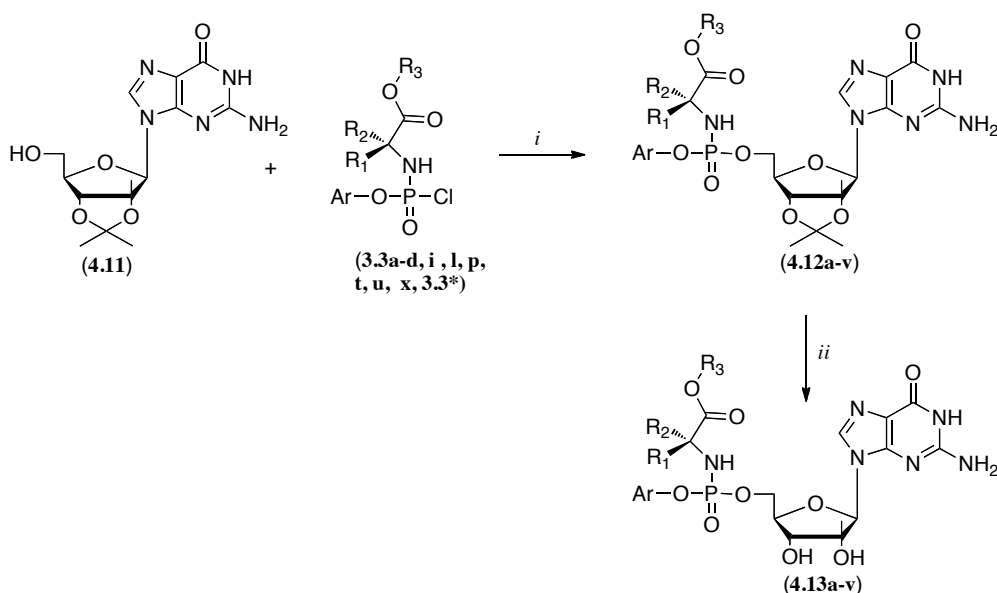


Reagents and Conditions: i) HClO_4 , dry acetone, rt, overnight.

Scheme 4.4. Synthesis of 2',3'-isopropylidene-2'-C- β -methylguanosine.

4.4 Synthesis of 2'CMeG phosphoramidates.

The phosphoramidate derivatives were obtained by the coupling of protected nucleoside with the appropriate phosphorochloridate in dry THF at ambient temperature, using *tert*-butylmagnesium chloride (*t*BuMgCl) as a base.¹⁵ Overnight reaction followed by purification on silica gel resulted in isopropylidene-protected intermediates **4.12a-v** (Scheme 4.5) in 30-90% yield.



Reagents and Conditions: i) *t*BuMgCl, dry THF, rt, overnight; ii) 60% acetic acid, 95 °C.

3.3*- phosphorochloridates available from the laboratory: α -naphthyl-(1-methoxy-2-propoxy-L-alaninyl)-; α -naphthyl-(*R,S*-phenylethoxy-L-alaninyl)-; β -naphthyl-(benzoxo-L-alaninyl)-; α -naphthyl-(*p*-chloro-benzoxo-L-valinyl)-; α -naphthyl-(*o*-methyl-benzoxo-L-valinyl)-; α -naphthyl-(benzoxo-L-isolucinyl) phosphorochloridate, phenyl-(benzoxo-L-alaninyl)-.

Scheme 4.5. General synthesis of 2'-C- β -methylguanosine ProTides.

Protected phosphoramidates were finally deprotected using 60% acetic acid solution in water at 95 °C overnight, to give final phosphoramidate ProTides **4.13a-v** (Scheme 4.5) in moderate to good yields (25%-58%).

The relative stability of the ProTide unit under these conditions confirms their surprising acid-stability and may play an important role for oral administration of these compounds.

The purified phosphoramidates were isolated as mixtures of two diastereoisomers at the phosphorus centre, confirmed by the presence of two peaks (in most cases) in the ^{31}P NMR and HPLC.

| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | Isomer ratio (NMR) | Isomer ratio (HPLC) |
|--------------|----------------|---|-----------------------------------|---------------------|-------------------------|--------------------|---------------------|
| 4.13a | Ph | CH ₃ | H | L-Ala | benzyl | 54:46 | 48:52 |
| 4.13b | α -Naph | CH ₃ | H | L-Ala | methyl | 48:52 | 38:62 |
| 4.13c | α -Naph | CH ₃ | H | L-Ala | <i>i</i> -propyl | 62:38 | 40:60 |
| 4.13d | α -Naph | CH ₃ | H | L-Ala | <i>t</i> -butyl | 52:48 | 46:54 |
| 4.13e | α -Naph | CH ₃ | H | L-Ala | <i>n</i> -butyl | 58:42 | 43:57 |
| 4.13f | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 57:43 | 31:69 |
| 4.13g | α -Naph | CH ₃ | H | L-Ala | cyclohexyl | 55:45 | 34:66 |
| 4.13h | α -Naph | CH ₃ | H | L-Ala | 1-methoxy-2-propyl | 28:32:40 | 31:11:58 |
| 4.13i | α -Naph | CH ₃ | H | L-Ala | benzyl | 53:47 | 45:55 |
| 4.13j | α -Naph | CH ₃ | H | L-Ala | <i>R,S</i> -phenylethyl | 54:28:18 | 33:14:35:18 |
| 4.13k | α -Naph | CH ₃ | H | L-Ala | <i>S</i> -phenylethyl | 60:40 | 42:58 |
| 4.13l | β -Naph | CH ₃ | H | L-Ala | benzyl | 50:50 | 43:57 |
| 4.13m | α -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 55:45 | 38:62 |
| 4.13n | α -Naph | H | CH(CH ₃) ₂ | D-Val | benzyl | 34:66 | 68:32 |
| 4.13o | β -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 56:44 | 56:44 |
| 4.13p | α -Naph | CH(CH ₃) ₂ | H | L-Val | <i>o</i> -Cl-benzyl | 58:42 | 33:67 |
| 4.13r | α -Naph | CH(CH ₃) ₂ | H | L-Val | <i>m</i> -Cl-benzyl | 13:30:27:30 | 31:25:44 |
| 4.13s | α -Naph | CH(CH ₃) ₂ | H | L-Val | <i>o</i> -Me-benzyl | 62:38 | 36:64 |
| 4.13t | α -Naph | CH ₃ | CH ₃ | Me ₂ Gly | benzyl | 65:35 | 31:69 |
| 4.13u | α -Naph | CH(CH ₃)CH ₂ CH ₃ | H | L-Ile | benzyl | 55:45 | 1 peak |
| 4.13v | α -Naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | benzyl | 62:38 | 36:64 |

AA: Amino acid.

Table 4.1. Summary of 2'CMeG ProTides and their isomer ratio determined by HPLC and NMR.

The approximate ratio of isomers determined by the HPLC (reverse phase system) is given in Table 4.1 and is very similar to the one given by ^{31}P NMR. Based on these findings, correlation between compound polarity and NMR shift was found. In most of the cases, main/major isomer peak had more downfield NMR shift, which corresponds to the less polar peak in the HPLC chromatogram (Figure 4.1).

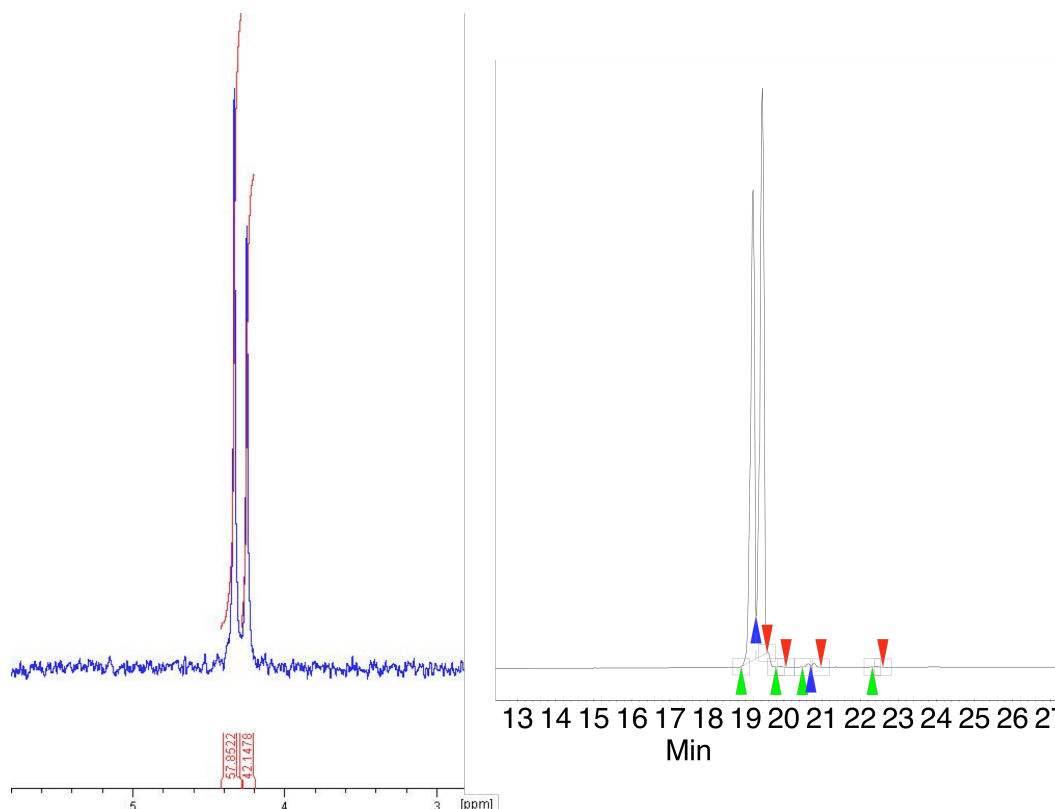


Figure 4.1. Comparison between isomer ratio of **4.13f** determined by ^{31}P NMR and HPLC (reverse phase system).

Considering HPLC retention times, all of the synthesised ProTides were more lipophilic than the parent nucleoside. Calculated lipophilicity values (ClogP)¹⁶ correspond to the HPLC data, and indicate up to 100-fold increase in lipophilicity. However, no clear correlation between calculated ClogP values and biological activity (EC_{50} values) of ProTides was found (Table 4.2-4.4).

The initial series of compounds was planned considering L-alanine as the primary amino acid centre and phenoxy or α -naphthyloxy group as an aryl moiety (Table 4.1 entries **4.13a-4.13k**). Several phosphoramidates containing different esters:

polar or lipophilic, linear or containing α and/or β branching were prepared to evaluate their effect on enzymatic lability and consequent influence on biological activity.

The subsequent series of compounds was designed for better understanding the structure activity relationships (SARs). Beside ester variation, the family consisted of ProTides bearing different amino acid moieties: L- and D-valine, L-isoleucine, L-methionine, and unnatural achiral amino acid dimethylglycine (Table 4.1, entries **4.13m-n**, **4.13p-v**). Most of them were prepared as benzyl esters except for methionine ProTide, which was synthesised as an isopropyl ester **4.13v** (Table 4.1). The aryl unit was also modified using phenol, α -naphthol or β -naphthol (Entries **4.13a**, **4.13l** and **4.13o** Table 4.1).

4.5 Biological evaluation of 2'-C- β -methylguanosine ProTides.

2'-C- β -methylguanosine ProTides were evaluated by Inhibitex Inc., against hepatitis C virus *in vitro* using a replicon assay. The cell line used for this assay was human hepatoma cell line (Huh 7).

The replicon used for assay consist of HCV 5'-NTR, the gene encoding luciferase (Re-Luc protein, obtained from *Renilla*), fused with neomycin phosphotransferase (NPTII) and a part of HCV genome containing NS3, NS4B, NS5A, NS5B and HCV 3'-NTR subunits.

After *in vitro* transcription replicon RNA strand is incorporated into the Huh cells. Neomycin resistant colonies were isolated and used for further tests.

Replicon potency

Huh7 cells expressing the HCV genotype were seeded into 96-well plates at density of 2×10^4 cell/well. Then 18-24 h after plating, inhibitors (synthesised ProTides) were added and cells were incubated for the additional 48 h.

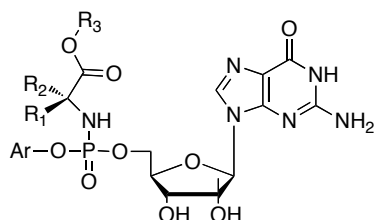
Compounds were tested in triplicates and quadruplicates at 3x or 4x serial dilutions over a range of 0.0001-10 μ M concentrations. HCV replication was monitored by Renilla luciferase reporter activity assay using Renilla luciferase reporter (Promega, Medison, WI) and a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Then 50% inhibitory concentration (EC₅₀) values were calculated as the concentration of

compound that results in 50% decrease in the reporter expression as compared to untreated cells. The values were determined by nonlinear regression analysis.

Cytotoxicity assay

The same cells used in the replicon assay were seeded into 96-well plates at density of 2×10^4 cells per well. 24 h after plating, 2x compound dilutions, starting with 100 μM , were applied to the testing plates (three repeats per each compound dilution). Each testing plate was incubated for 72 h. To determine cell viability, the CellTiter-Glo assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. The compound concentration resulting in 50% luminescent signal was reported as the CC_{50} concentration.

The phosphoramidate technology allowed to convert a nucleoside with μM activity into ProTides with nM activity (Tables 4.2-4.4).



| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ (μM) | CC ₅₀ (μM) |
|--------------|----------------|-----------------|----------------|-------|-------------------------|-------|------------------------------------|------------------------------------|
| 4.10 | - | - | - | - | - | -2.6 | 3.5 | >100 |
| 4.13a | Ph | CH ₃ | H | L-Ala | benzyl | 0.02 | 0.16 | >100 |
| 4.13b | α -Naph | CH ₃ | H | L-Ala | methyl | -0.5 | 0.21 | >50 |
| 4.13c | α -Naph | CH ₃ | H | L-Ala | <i>i</i> -propyl | 0.3 | 0.17 | >100 |
| 4.13d | α -Naph | CH ₃ | H | L-Ala | <i>t</i> -butyl | 0.7 | >20 | >100 |
| 4.13e | α -Naph | CH ₃ | H | L-Ala | <i>n</i> -butyl | 1.1 | 0.10 | >100 |
| 4.13f | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 1.3 | 0.05 | >100 |
| 4.13g | α -Naph | CH ₃ | H | L-Ala | cyclohexyl | 1.5 | 0.05 | >100 |
| 4.13h | α -Naph | CH ₃ | H | L-Ala | 1-methoxy-2-propyl | -0.10 | 0.13 | >100 |
| 4.13i | α -Naph | CH ₃ | H | L-Ala | benzyl | 1.2 | 0.06 | >100 |
| 4.13j | α -Naph | CH ₃ | H | L-Ala | <i>R,S</i> -phenylethyl | 1.5 | 0.09 | >100 |
| 4.13k | α -Naph | CH ₃ | H | L-Ala | <i>S</i> -phenylethyl | 1.5 | 0.08 | >100 |
| 4.13l | β -Naph | CH ₃ | H | L-Ala | benzyl | 1.2 | 0.17 | >50 |
| 1.1 | - | - | - | - | - | -2.8 | 33.7 | ~60 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration;

Table 4.2. Summary of 2'CMcG L-alanine ProTides.

Among the L-alanine series of 2'CMeG phosphoramidates, the least active were compounds bearing methyl **4.13b** and *tert*-butyl **4.13d** esters (Table 4.2 and Figure 4.2). For the methylalanine ester **4.13b** a low antiviral activity may correspond to the low calculated ClogP value -0.5. Indicating that lipophilicity of this compound was lower than that considered as an optimal for passive cellular uptake. The potency of **4.13d** was found to be lower than the parent nucleoside. Lower EC₅₀ value of *tert*-butyl ester may be related to the relative stability of this ester to the enzyme-mediated hydrolysis, during the first step of phosphoramidate activation, resulting in lack of efficient liberation of 5'-monophosphate.

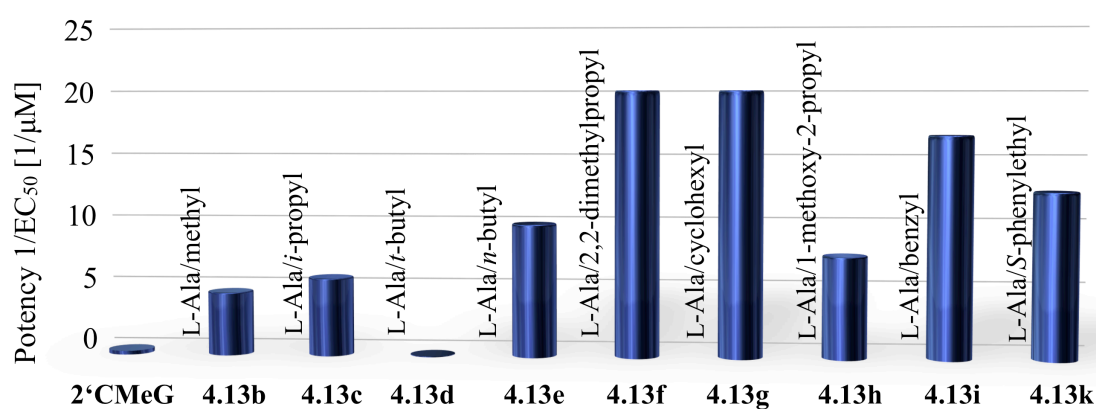
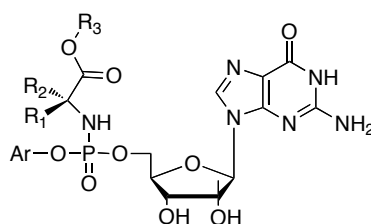


Figure 4.2. Potency (1/EC₅₀) data plot of L-alanine ProTides of 2'CMeG.

Further lengthening and branching of the ester unit in L-alanine series resulted in compounds like **4.13f** and **4.13g** with EC₅₀=0.05 μM. These compounds were amongst the most active derivatives across the family. Introduction of an additional chiral centre in ester moiety **4.13h** and **4.13j** gave a mixture of four diastereoisomers, confirmed by ³¹P NMR and HPLC, and in case of 1-methoxy-2-propyl ester **4.13h** a relative decrease in antiviral activity (comparing to the most active ProTides). Aromatization of the ester led to the compounds such as L-alanine benzyl ester **4.13i** with a calculated ClogP=1.2 and its branched analogues **4.13j** (*R,S*-phenylethyl L-alanine) and **4.13k** (*S*-phenylethyl L-alanine) showing nanomolar activity in replicon assay.

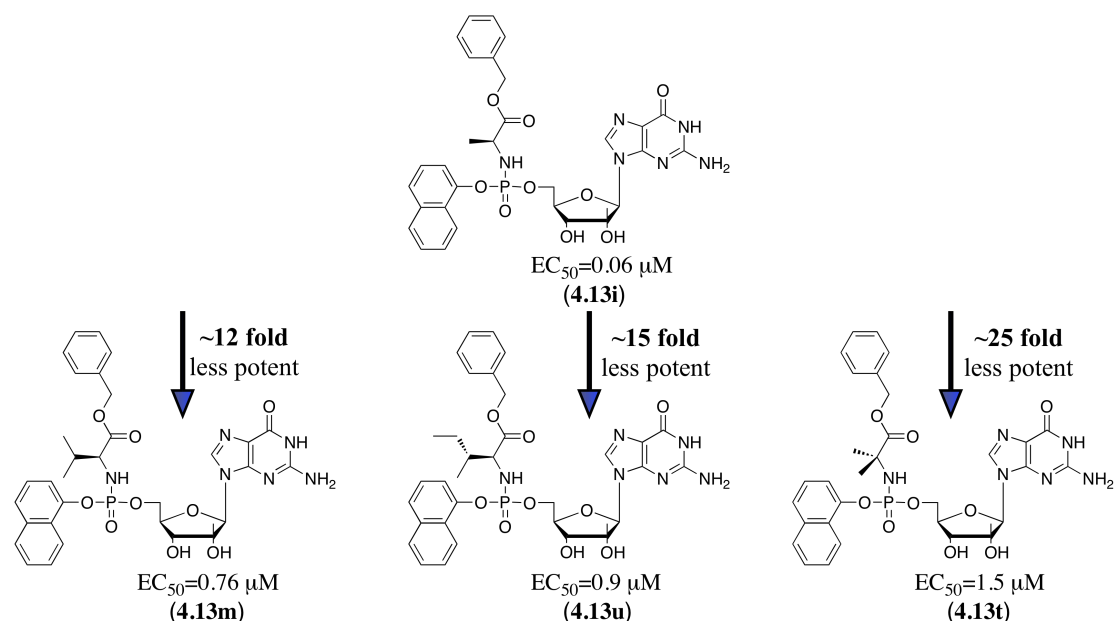


| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ (μM) | CC ₅₀ (μM) |
|--------------|--------|---|-----------------------------------|---------------------|------------------------|-------|-----------------------|-----------------------|
| 4.10 | - | - | - | - | - | -2.6 | 3.5 | >100 |
| 4.13m | α-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 2.1 | 0.76 | >100 |
| 4.13n | α-Naph | H | CH(CH ₃) ₂ | D-Val | benzyl | 2.1 | >3 | >100 |
| 4.13o | β-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 2.1 | 1.7 | >100 |
| 4.13p | α-Naph | CH(CH ₃) ₂ | H | L-Val | <i>o</i> -Cl-benzyl | 2.8 | 0.43 | >100 |
| 4.13r | α-Naph | CH(CH ₃) ₂ | H | L-Val | <i>m</i> -Cl-benzyl | 2.8 | 0.68 | >100 |
| 4.13s | α-Naph | CH(CH ₃) ₂ | H | L-Val | <i>o</i> -Me-benzyl | 2.6 | 1.0 | >100 |
| 4.13t | α-Naph | CH ₃ | CH ₃ | Me ₂ Gly | benzyl | 1.5 | 1.5 | >100 |
| 4.13u | α-Naph | CH(CH ₃)CH ₂ CH ₃ | H | L-Ile | benzyl | 2.7 | 0.9 | >100 |
| 4.13v | α-Naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | isopropyl | 1.3 | 0.34 | >100 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 4.3. Summary of 2'CMeG ProTides bearing different amino acid ester units.

Structure activity relationships of a 2'-C-methylguanosine ProTides bearing different amino acid esters is presented in Table 4.3. Bulky and lipophilic amino acids were chosen to increase logP of these compounds and therefore enhance cellular uptake. Additionally compounds were considered as more stable towards enzymatic degradation. All compounds in general were less active than L-alanine analogues, showing ca. 5-25-fold decrease in potency. Comparing activity of ProTides **4.13m-v** it was observed that disubstitution of the α-carbon lead to 25-fold decrease in potency (**4.13t** vs **4.13i**). Branching at β-carbon **4.13m** (L-valine) gave 4-fold improvement in potency comparing to the 2'CMeG but ~12-fold decrease of activity in respect to the lead L-alanine benzyl ester **4.13i** (Scheme 4.6).



Scheme 4.6. Comparison of EC_{50} of different 2'CMcG ProTides. EC_{50} values in μM .

Longer amino acids e.g. L-Met **4.13v** kept submicromolar activity and the compound was found to be equipotent with the corresponding L-alanine derivative **4.13c**.

Introduction of D-amino acid **4.13n** resulted in loss of activity in case of both alanine and valine derivatives.

The SAR of aryloxy units reported in Table 4.4. Comparison between α -naphthol and phenol shows 2.5-fold decrease in antiviral potency when the latter was used. This difference can be explained by increase in lipophilicity and leaving group ability. When α -naphthol was replaced by β -naphthol, ~ 3 -fold decrease in activity was observed for both L-alanine and L-valine families.

| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC_{50} (μM) | CC_{50} (μM) |
|--------------|----------------|-----------------------------------|----------------|-------|------------------------|-------|-----------------------------|-----------------------------|
| 4.10 | - | - | - | | - | -2.6 | 3.5 | >100 |
| 4.13a | Ph | CH ₃ | H | L-Ala | benzyl | 0.02 | 0.16 | >100 |
| 4.13i | α -Naph | CH ₃ | H | L-Ala | benzyl | 1.2 | 0.06 | >100 |
| 4.13l | β -Naph | CH ₃ | H | L-Ala | benzyl | 1.2 | 0.17 | >100 |
| 4.13m | α -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 2.1 | 0.76 | >100 |
| 4.13o | β -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 2.1 | 1.7 | >100 |

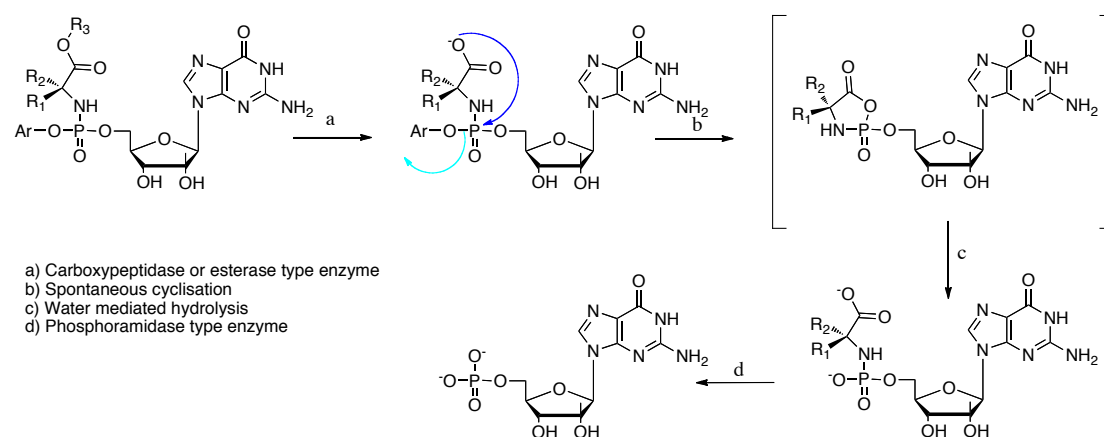
AA: Amino acid; EC_{50} : 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC_{50} : 50% cytotoxic concentration.

Table 4.4. Summary of 2'CMcG ProTides bearing different amino acid ester units.

All of the synthesised ProTides displayed minimal toxicity in the Huh7 cells. CC_{50} values were usually greater than 100 μ M, providing compounds with very high selectivity indexes. Several compounds were also tested in MT4, CEM, HepG2, HEL and Caco2 cell lines. Similarly no significant toxicities were found.

4.6 Enzymatic studies using Carboxypeptidase Y on 2'CMeG ProTides.

The putative mechanism of activation of phosphoramidate prodrugs as already described in Chapter 2, requires an initial hydrolysis of the ester unit by esterase or carboxypeptidase type enzyme. Subsequently the nucleophilic attack of the carboxyl group on phosphorus centre resulting in spontaneous cyclisation and elimination of the aryloxy moiety. A presumed five-membered ring is considered as very unstable and undergoes opening to give an intermediate diacid metabolite. The last step of bioactivation involves phosphoramidase-mediated hydrolysis of P-N bond to obtain the desired monophosphate. It is postulated that this deamination process relies on human histidine triad nucleotide binding protein 1 (HINT1) activity (Scheme 4.7).¹⁵ The resulting monophosphate can be then phosphorylated to the corresponding di- and triphosphate by specific kinases.



Scheme 4.7. Putative mechanism of activation of 2'CMeG ProTides.

In the work carried out by Birkus *et al.*¹⁷ it was discovered that one of the lysosomal associated enzymes – cathepsin A (carboxypeptidase A, CatA,

EC 3.4.16.5) was responsible for the ester cleavage during activation pathway of nucleotide amidate prodrugs.¹⁷

Cathepsin A is a member of α/β -fold hydrolase family. It is multifunctional enzyme with carboxypeptidase, esterase and deamidase catalytic activity.¹⁸ In terms of substrate specificity; CatA shows high affinity for hydrophobic and basic amino acids. Second enzyme that could be involved in the cleavage of the carboxyester moiety is carboxylesterase 1 (liver carboxylesterase 1, hCE-1, CES1, CES1A1, EC 3.1.1.56, EC 3.1.1.1). hCE-1 belongs to the same as CatA family of α/β -fold hydrolases.¹⁹ This enzyme was found to efficiently hydrolyse amide, ester and thioester bonds in aromatic and aliphatic substrates.²⁰

Tissue distribution assays demonstrated that both of these enzymes have high levels of expression in variety of tissues, especially in kidneys, liver and lungs.²¹ High levels of these enzymes present in the liver may lead to ProTides being processed more efficiently in hepatocytes, which could result in increased efficacy and at the same time reduced systemic exposure and cytotoxicity.

To better understand the influence of different esters on potency and antiviral activity of 2'-C-methylguanosine aryloxyphosphoramidates, ester hydrolysis studies were performed. As human CatA is not commercially available; carboxypeptidase Y was chosen as a model enzyme. Carboxypeptidase Y (CPY, EC 3.4.16.1) is a yeast carboxypeptidase exhibiting a high degree of structural homology around the catalytic site with cathepsin A. In terms of substrate specificity, the two enzymes belong to the same family of C-type carboxypeptidases.²²

The procedure used for enzymatic studies was developed within the McGuigan group.²³ The experiments were run at 25 °C, optimal temperature for carboxypeptidase Y hydrolytic activity, and at pH 7.6, the enzyme reaction was followed by ³¹P NMR. Initially, the blank ³¹P NMR of the appropriate phosphoramidate in acetone-*d*₆ and TRIZMA buffer (pH 7.6) was recorded. Then compound was incubated with carboxypeptidase Y for period of 14 h and the ³¹P NMR spectra were recorder every 7 minutes.

Described in the following section are the enzymatic assays performed on the 2'CMeg phosphoramidate derivatives reported in the previous section of this Chapter.

A first experiment was performed with the α -naphthyl L-alanine benzyl ester 5'-phosphoramidate **4.13i**. In the blank ^{31}P NMR two diastereoisomers (1:1 ratio) signals were observed ($\delta_{\text{P}} = 3.69$ and 4.12 ppm).

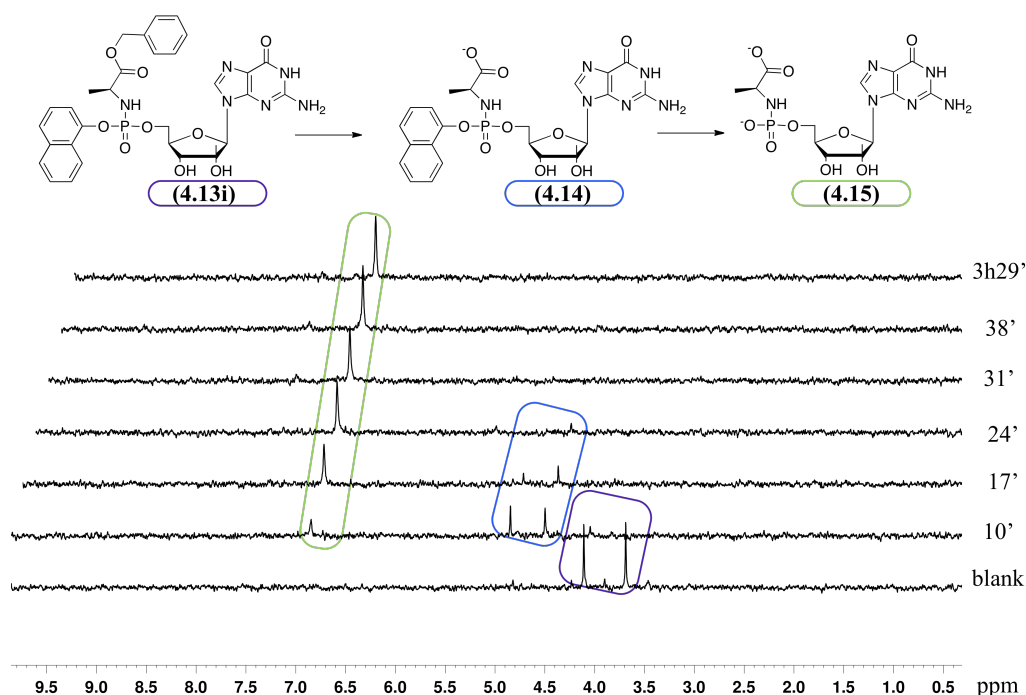


Figure 4.3. Carboxypeptidase-mediated cleavage of compound **4.13i**, monitored by ^{31}P NMR.

Once the enzyme was added, compound was quickly hydrolysed to the intermediate **4.14** ($\delta_{\text{P}} = 4.53$ and 4.85 ppm), which appeared in 1:1 ratio. At the 7-minute time point, a peak corresponding to the final aminoacyl intermediate **4.15** ($\delta_{\text{P}} = 6.98$ ppm) was formed (Figure 4.3). The half-life of the compound is ca. 3 min.

In the case of the isopropyl derivative **4.13c**, the compound was found to be well processed. Interestingly, one of the **4.13c** diastereoisomers was hydrolysed much faster, presumably indicating that this isomer fits better in the active site of the enzyme. However, it has to be noted that the ratio of isomers in the starting phosphoramidate **4.13c** was roughly 2:1 and it also might have an impact on the rate of processing. Like in the case of benzyl derivative, the final product has similar δ_{P} of 6.95 ppm, as both compounds are activated to the same aminoacyl intermediate. Half-life of the compound was found to be 20 min (Figure 4.4).

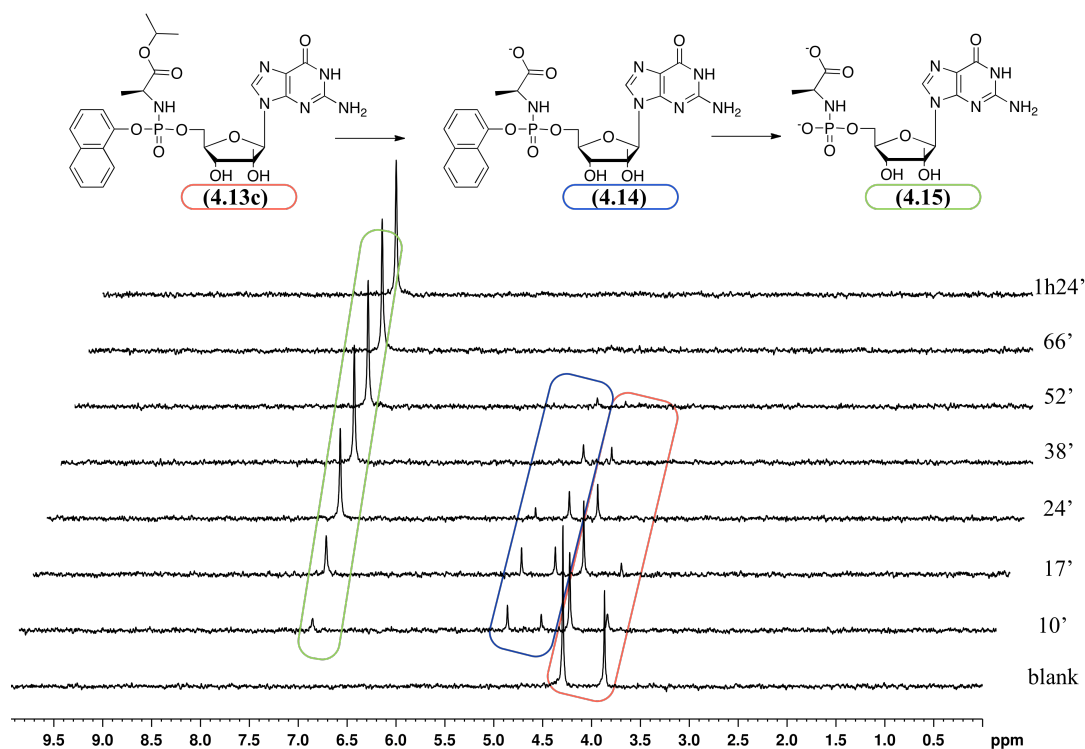


Figure 4.4. Carboxypeptidase-mediated cleavage of compound **4.13c**, monitored by ^{31}P NMR.

By contrast, L-alanine *tert*-butyl derivative **4.13d** was found to be processed very slowly (Figure 4.5).

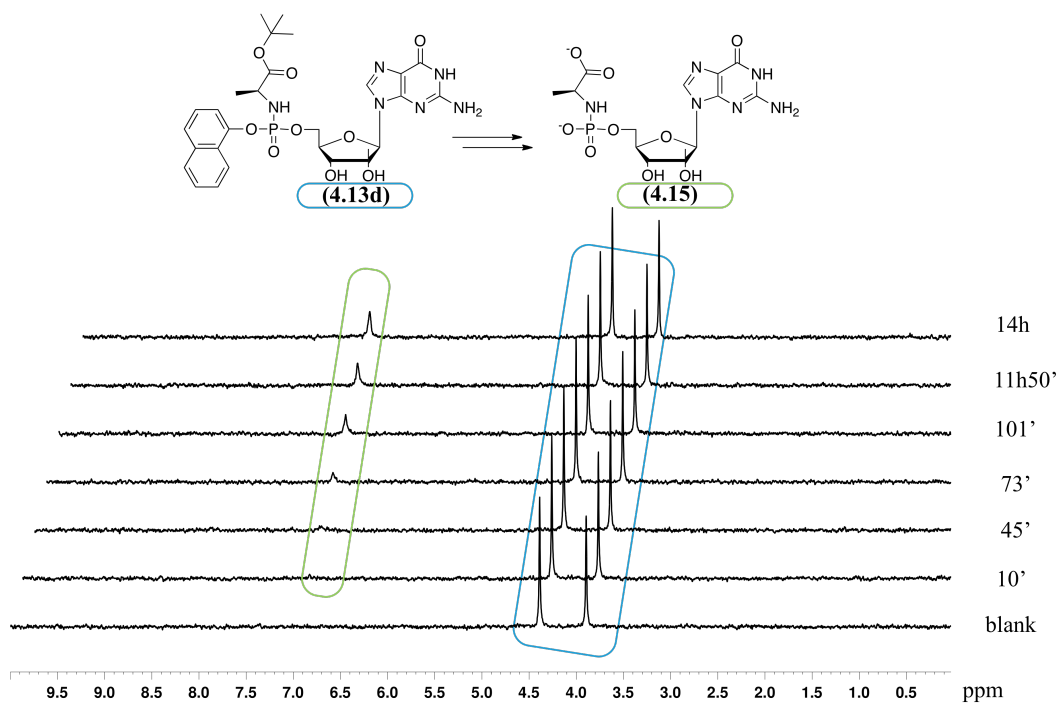


Figure 4.5. Carboxypeptidase-mediated cleavage of compound **4.13d**, monitored by ^{31}P NMR.

Final intermediate peak **4.15** started to form within 1.5 hour after enzyme addition, and estimated half-life of the compound was 50 h (Figure 4.5).

The experiment carried out with *t*-butyl ester phosphoramidate is in accordance with the lack of antiviral activity. As already mentioned *t*-butyl ester is probably too bulky to be processed by the enzyme and therefore 5'-monophosphate is not efficiently released resulting in deficiency in antiviral activity.

The carboxypeptidase assay performed on **4.13m** shows good conversion of the starting ProTide (Figure 4.6).

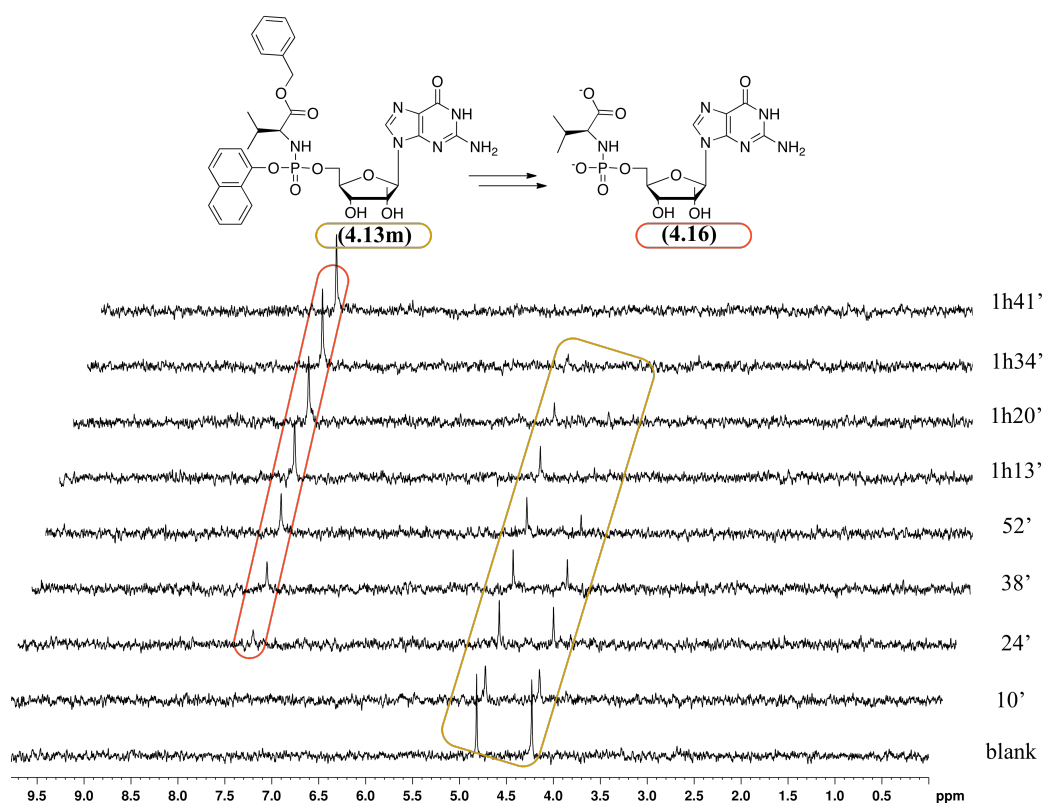


Figure 4.6. Carboxypeptidase-mediated cleavage of compound **4.13m**, monitored by ^{31}P NMR.

24 min after addition of carboxypeptidase, appearance of new peak at $\delta_{\text{P}} = 7.49$ ppm was observed. This new species corresponds to the final L-valinyl intermediate **4.16** (Figure 4.6). Comparing to the analogous L-alanine derivative **4.13i**, processing of **4.13m** is slower ($T_{1/2} \sim 30$ min vs. 7 min) and it is consistent with activity found for these two compounds ($\text{EC}_{50} = 0.76$ vs. $0.06 \mu\text{M}$).

On the other hand D-Val analogue **4.13n** was found to be both, not processed by carboxypeptidase and not active (Figure 4.7).

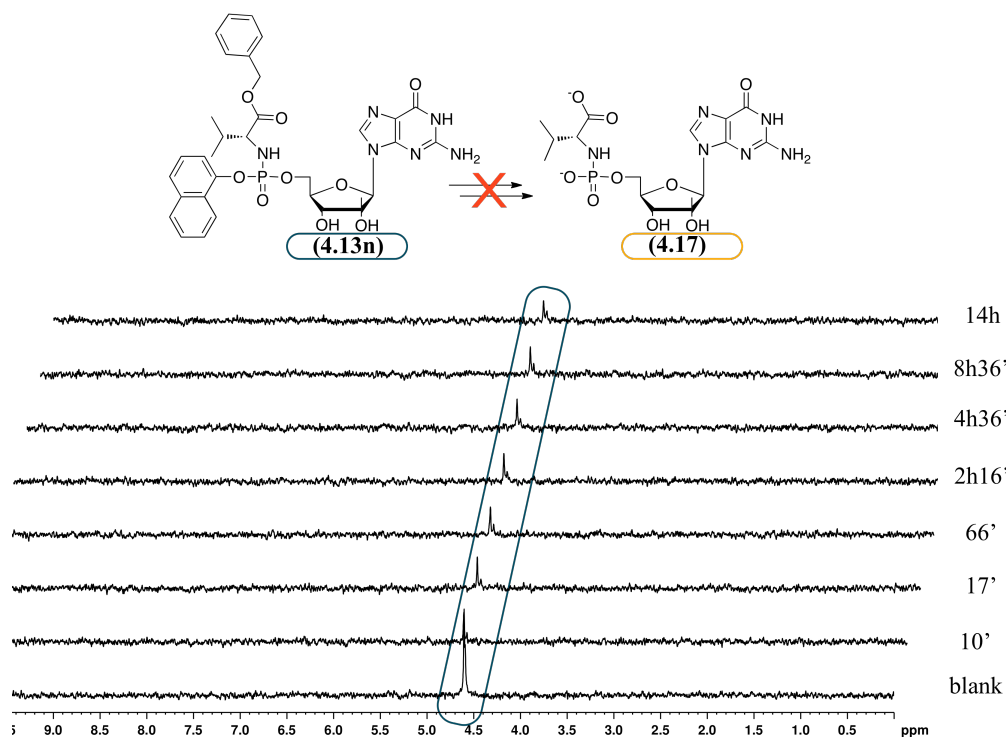


Figure 4.7. Carboxypeptidase-mediated cleavage of compound **4.13n**, monitored by ^{31}P NMR.

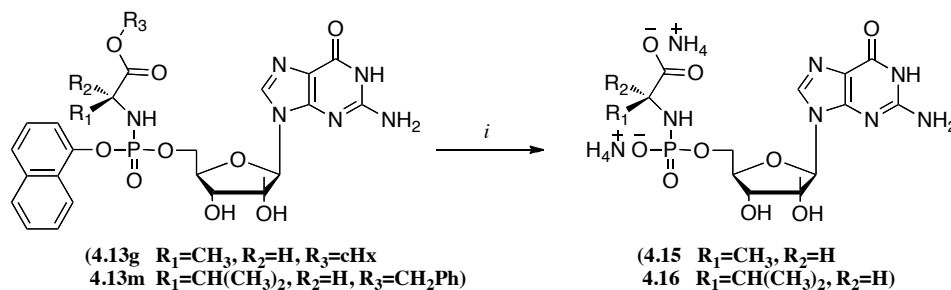
All carboxypeptidase Y assays should be considered as a model prediction of phosphoramidate activation. It may be possible that *in vivo* activation of particular phosphoramidate examples will differ (presence of various enzymes being potentially involved in ester cleavage) and may result in faster or slower release of the corresponding aminoacyl intermediates.

4.7 Synthesis of metabolic intermediates.

In order to verify chemical structure of final metabolic intermediates present during carboxypeptidase Y assays, synthesis of **4.15** and **4.16** was performed (Scheme 4.8). Subsequently ^{31}P NMR shifts of synthesised compounds were compared to those obtained through enzymatic cleavage.

Synthesis of diacid intermediates **4.15** and **4.16** involved hydrolysis of previously prepared phosphoramidates **4.13g** and **4.13n**, respectively. Appropriate phosphoramidates were dissolved in a 1:1 mixture of triethylamine and water,²⁴ and

reaction mixtures were stirred at room temperature for 16 h (Scheme 4.8). After column chromatography, products were obtained as diammonium salts. Structure of both compounds was confirmed by NMR analysis and mass spectroscopy.



Reagents and Conditions: i) $\text{Et}_3\text{N}:\text{H}_2\text{O}$ (1:1), rt, 16 h.

Scheme 4.8. General synthesis of 2'-C- β -methylguanosine aminoacyl intermediates.

Synthesised intermediates were dissolved in acetone- d_6 and TRIZMA buffer, to mimic conditions used for carboxypeptidase Y experiments, and ^{31}P NMR spectra were recorded (Figure 4.8).

In the spectrum recorded during carboxypeptidase Y assay using compound **4.13i**, after complete processing, only one single peak can be seen at $\delta_{\text{P}} = 6.99$ ppm, which could correspond to the achiral phosphoramidate monoester intermediate (Figure 4.8a). Spectra of chemically prepared 5'-aminoacyl diammonium salt of **4.15** shows also one peak at $\delta_{\text{P}} = 6.93$ ppm (Figure 4.8b), and as can be seen both peaks coincide. To further support these findings ^{31}P NMR of both compounds together, were recorded and analysed (Figure 4.8c). It can be clearly noticed that both peaks overlap, without any ^{31}P signal splitting being observed. These results strongly support the structure of the aminoacyl intermediate present during enzymatic activation of L-alaninyl phosphoramidates of 2'CMcG.

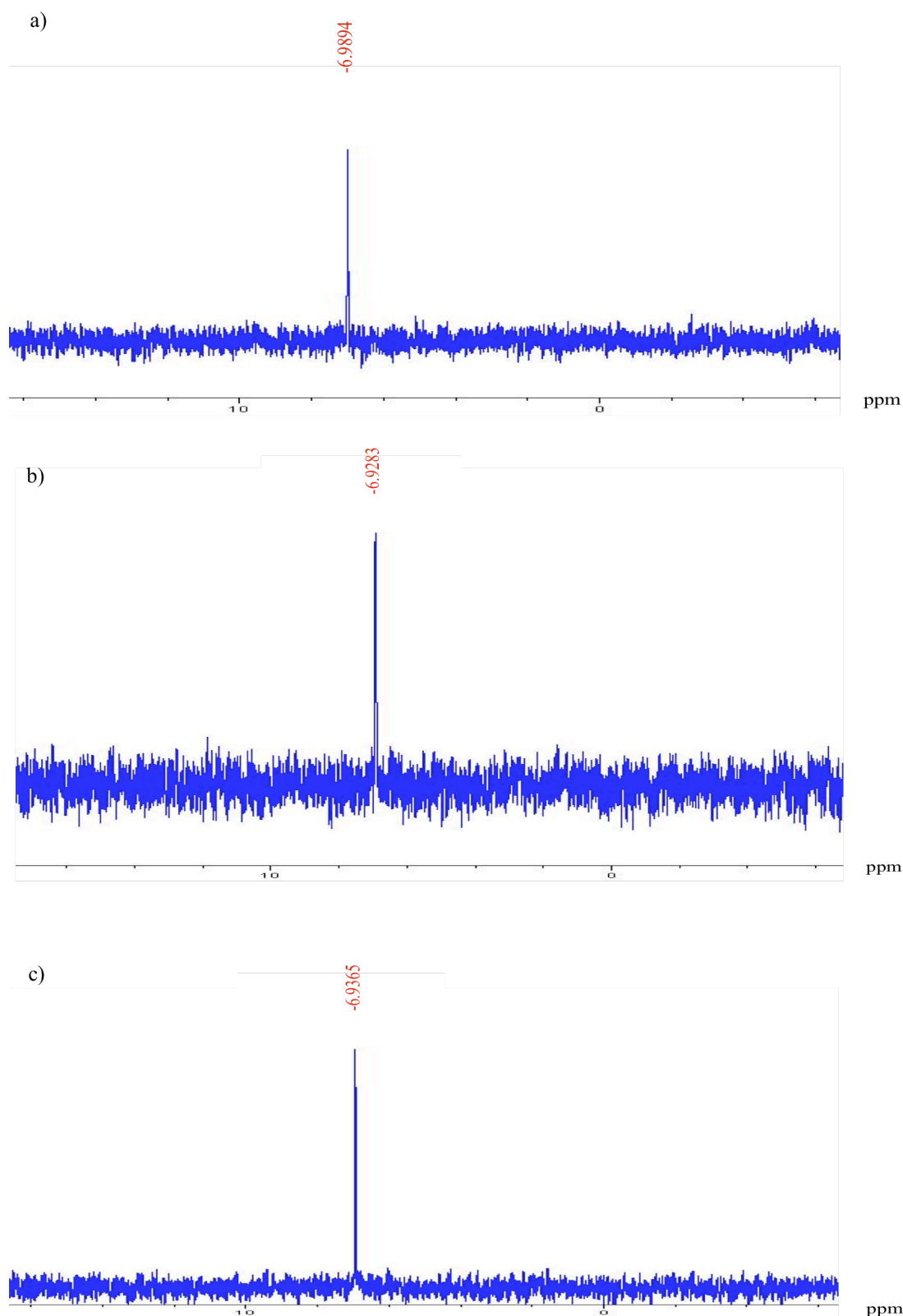
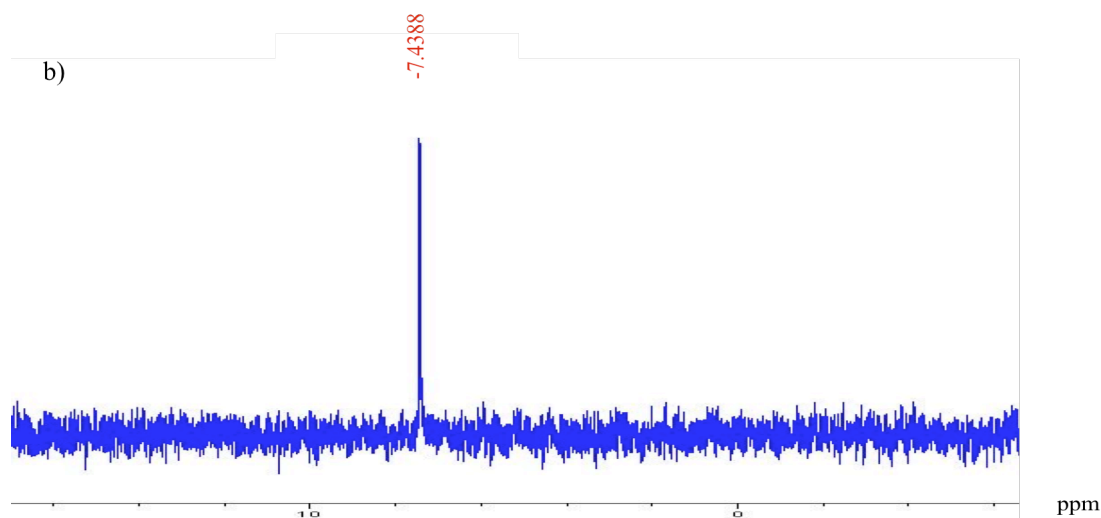
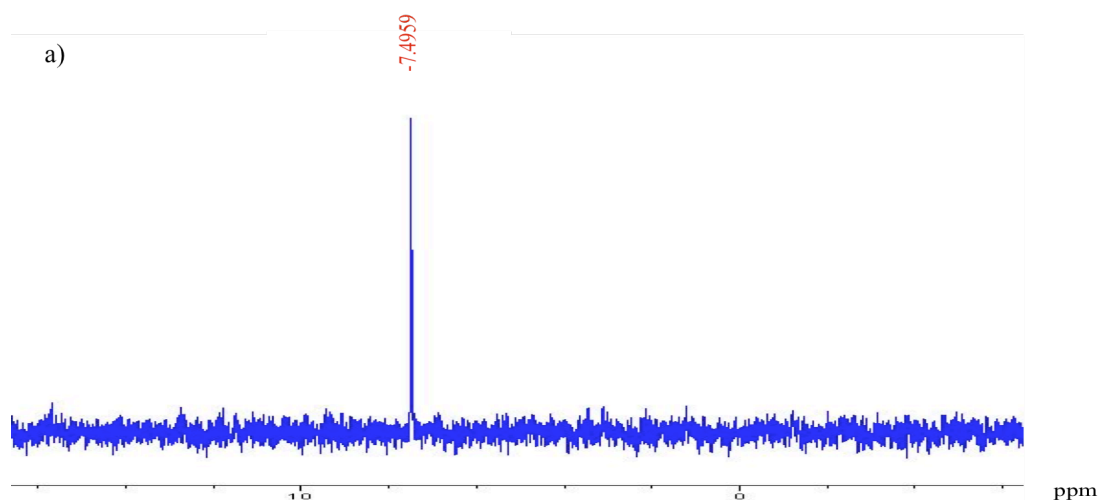


Figure 4.8. ^{31}P NMR analysis of metabolic intermediate observed during carboxypeptidase-mediated cleavage of phosphoramidates; a) ^{31}P NMR spectra of phosphoramidate **4.13i** submitted for enzyme assay, b) ^{31}P NMR spectra of synthesised L-alaninyl intermediate **4.15**, c) ^{31}P NMR spectra of a mixture of enzymatic **4.13i** and **4.15**.

A similar methodology was applied to L-valine derivatives in order to verify the structure of the intermediate present during carboxypeptidase assay performed on **4.13m**. After complete enzymatic conversion of the L-valinyl phosphoramidate **4.13m** to the proposed corresponding diacid intermediate **4.15**, the ^{31}P NMR spectrum was recorded (Figure 4.9a). Like in the case of L-alaninyl analogue, one single peak was observed with chemical shift of 7.49 ppm.

When the chemically prepared compound was submitted to the NMR analysis (Figure 4.9b), one single peak at $\delta_{\text{P}} = 7.44$ ppm was observed. After mixing two samples together, both peaks overlap resulting in one ^{31}P signal, with chemical shift of 7.46 ppm (Figure 4.9c).



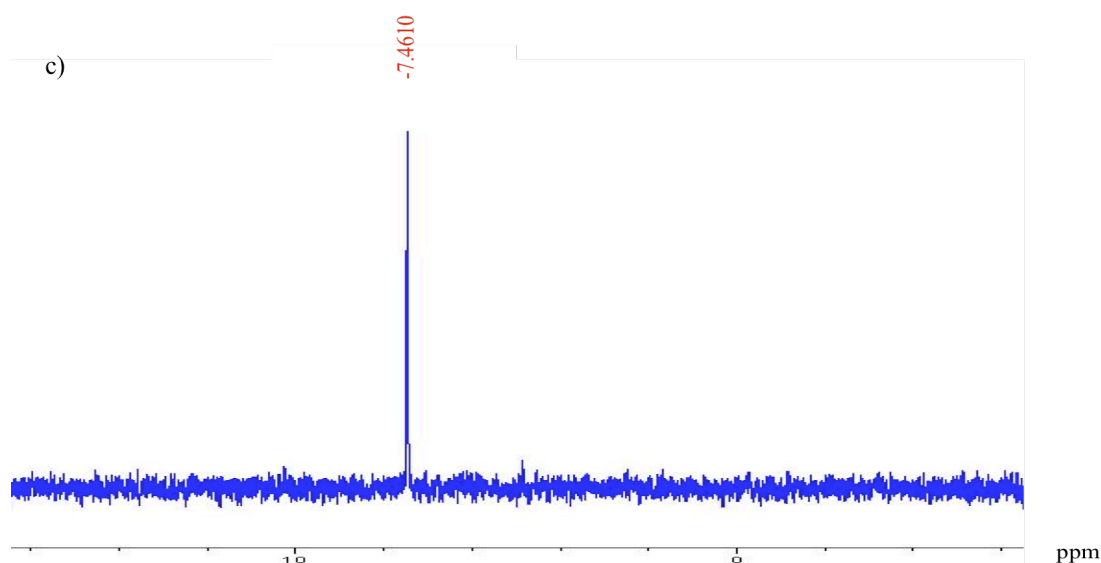


Figure 4.9. ^{31}P NMR analysis of metabolic intermediate observed during carboxypeptidase-mediated cleavage of phosphoramidates; a) ^{31}P NMR spectra of phosphoramidate **4.13m** submitted for enzyme assay, b) ^{31}P NMR spectra of synthesised L-alaninyl intermediate **4.16**, c) ^{31}P NMR spectra of a mixture of enzymatic **4.13m** and **4.16**.

Performed analyses gave an evidence, that compounds **4.15** and **4.16** formed during enzymatic assays and their equivalents prepared by chemical hydrolysis share the same molecular structure.

4.7.1 Biological evaluation of phosphoramidate monoesters of 2'CMcG.

Both synthesised 5'-L-alaninyl and 5'-L-valinyl monoester phosphoramidates (**4.15** and **4.16** respectively) were submitted for biological testing in HCV replicon assay. Results are reported in Table 4.5.

| Cpd | R1 | R2 | AA | EC ₅₀ (μM) | CC ₅₀ (μM) |
|-------------|-----------------------------------|----|-------|-----------------------|-----------------------|
| 4.10 | - | - | - | 3.5 | >100 |
| 4.15 | CH ₃ | H | L-Ala | 3.4 | >100 |
| 4.16 | CH(CH ₃) ₂ | H | L-Val | 6 | >100 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 4.5. Replicon activity of 2'CMcG aminoacyl intermediates.

As one might expect, based on their polarity, no improvement in antiviral potency comparing to the parent nucleoside **4.10** has been seen for both of these

synthesised compounds. However, the fact that moderate activity for this class of molecules has been seen at all is somewhat surprising. It can be explained that amino acid phosphate moiety is being cleaved outside the cell and only nucleoside is being delivered intracellularly. Another hypothesis is that the aminoacyl intermediates can in some extent facilitate cellular uptake and deliver monophosphate inside the cell, though the whole process is not as efficient as for the phosphoramidate ProTides.

4.8 Stability of 2'CMeG ProTides.

4.8.1 Plasma stability.

Replicon results revealed several interesting compounds, which could potentially be tested *in vivo*, in small animal models. Prior to animal pharmacokinetic studies, plasma stability was investigated at Inhibitex Inc. Data are % remaining in the plasma after 30 min of incubation (Figure 4.10 and 4.11).

It was clearly noticed that in terms of L-alanine phosphoramidates, all tested compounds were very unstable in rodent plasmas (both rat and mouse). Simple alkyl L-alanine ester ProTides **4.13b** (methyl) and **4.13e** (butyl) were among the least stable in rodent plasma, and were rapidly degraded. The most stable compound across this family was L-alanine isopropyl derivative **4.13c** exhibiting moderate stability in mouse plasma with 17% remaining after 30 min of incubation. Surprisingly, other branched esters like **4.13f** or **4.13k** did not improve rodent plasma stability and were almost completely cleaved within 30 min (Figure 4.10).

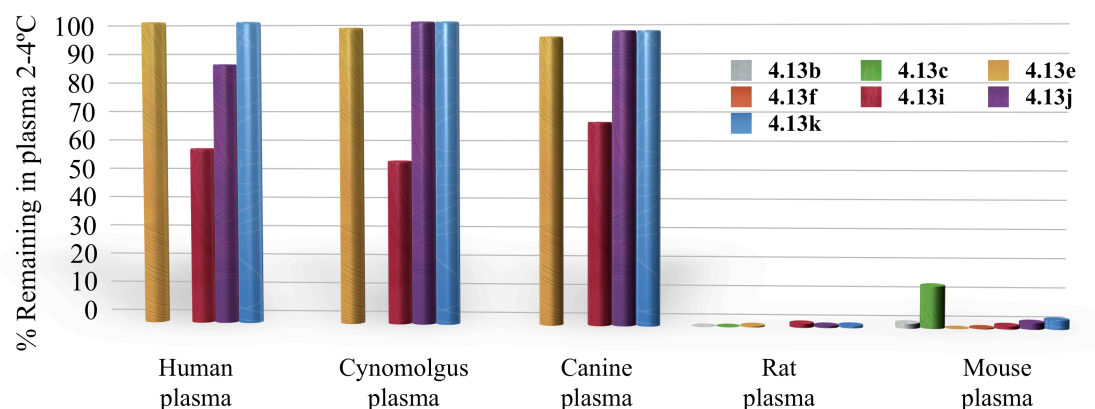


Figure 4.10. Plasma stabilities of selected phosphoramidates bearing different amino acid esters.

This profound plasma instability of L-alanine ProTides excludes these compounds as potential candidates for rodent pharmacokinetic studies.

On the other hand, most of the tested L-alanine compounds showed desirable stability in monkey and human plasma. The only exemption was compound **4.13i** (L-alanine benzyl ester), which was the least stable in human plasma with approximately 60% remaining after 30 min of incubation (Figure 4.10).

Considering compounds derived from different amino acids, it was noticed that compounds with disubstitution at alpha carbon e.g. **4.13t** (Figure 4.11) did not provide any improvement in terms of mouse or rat plasma stability, comparing to the corresponding L-alanine analogue. However, compounds bearing branching at beta carbon resulted in significantly improved plasma stability. Thus, L-valine derivatives exhibited considerably less rodent plasma instability than other amino acids. Interestingly, when L-valine **4.13m** and D-valine **4.13n** were evaluated for their stability in mouse plasma, **4.13n** was found to show a higher degree of instability than its L-analogue. These findings do not correlate with replicon results, where D-valine ProTide showed no antiviral activity, presumably because of the lack of activation.

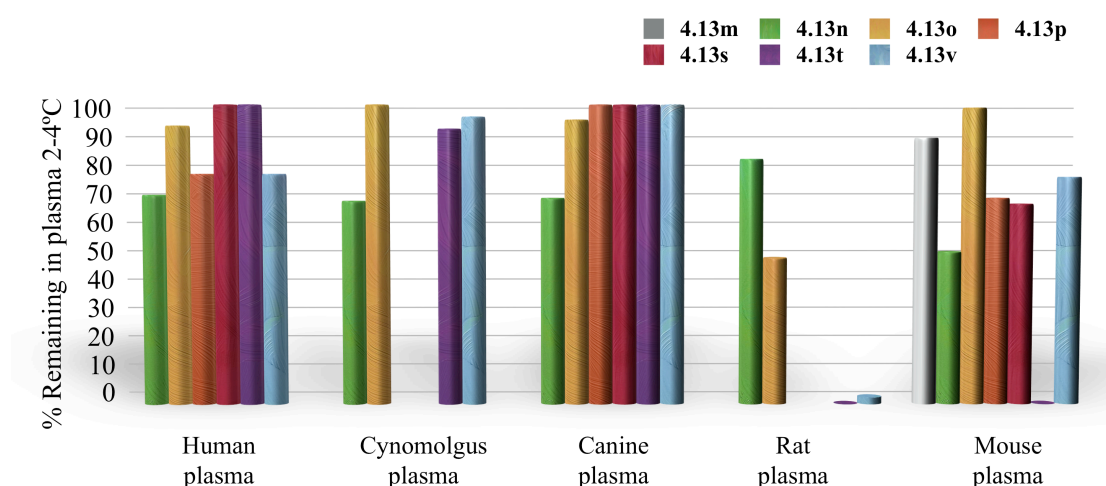


Figure 4.11. Plasma stabilities of selected L-alanine phosphoramidates.

Furthermore, compounds bearing substitutions in ortho position of benzyl ester **4.13p** and **4.13s** were slightly less stable than unsubstituted derivatives **4.13m** and **4.13o**. In terms of stability in human and monkey plasma, all compounds showed satisfactory stability.

4.8.2 Liver and intestinal S9 stability.

Liver and intestinal S9 stability studies across different species (human, monkey, dog and rat) were investigated in order to establish which species best maps onto the human profile of intestinal to liver stability. Additionally, compounds that were exhibiting high stability profile in intestinal S9 extracts with rapid cleavage in the liver were desirable, as they are considered as liver targeting. Compounds were incubated in the presence or without NADPH co-factor (Table 4.6), for 60 min (unless otherwise stated) at 37 °C. Reaction mixtures were quenched by addition of equal amounts of cold acetonitrile. After centrifugation (3000 rpm for 15 min), supernatant was submitted to HPLC analysis. Experiments were performed and evaluated by Inhibitex Inc.

| Cpd | | 4.13c | 4.13f | 4.13i | 4.13m | 4.13u |
|--|-----------------|------------------|--------------------|--------------------|-----------------------------------|---|
| Ar | | α -Naph | α -Naph | α -Naph | α -Naph | α -Naph |
| R ₁ | | CH ₃ | CH ₃ | CH ₃ | CH(CH ₃) ₂ | CH(CH ₃) ₃ CH ₂ CH ₃ |
| R ₂ | | H | | H | H | H |
| R ₃ | | <i>i</i> -propyl | 2,2-dimethylpropyl | benzyl | benzyl | benzyl |
| Intestinal and Liver S9 stability (% Remaining after 60 min of incubation) | | | | | | |
| Human | Intestine \pm | 65/85 | 28/49 | 0/49 ^a | 24/70 | 52/74 |
| | Liver \pm | 27/41 | 7/59 | 27/41 ^a | 0/78 | 29/83 |
| Monkey | Intestine \pm | 48/87 | 20/68 | 0/30 ^a | 25/77 ^a | 29/95 |
| | Liver \pm | 0/86 | 16/88 | 0/32 ^a | 0/77 ^a | 0/89 ^a |
| Dog | Intestine \pm | 66/68 | 42/56 | 8/29 | 56/72 | 87/96 |
| | Liver \pm | 81/91 | 49/87 | 16/53 | 56/75 | 64/84 |
| Rat | Intestine \pm | 77/76 | 57/86 | 30/46 ^a | 65/81 | 71/70 |
| | Liver \pm | 25/28 | 5/31 | 0/0 ^a | 8/62 | 37/78 |

\pm : with/without NADPH co-factor.

^a: incubated for 30 min.

Table 4.6. Intestinal and liver S9 stability of 2'CMcG ProTides.

Comparing stability data derived from intestinal and liver S9 preparations, it was observed that majority of the compounds exhibited greater stability in intestinal extracts than in the ones obtained from liver (Table 4.6). These findings give support to liver targeting by these ProTides. Across all tested compounds L-alanine isopropyl ProTide **4.13c** was found to be the most stable in both types of extracts, while benzyl analogues in general (**4.13i**, **4.13m**, **4.13u**) were more prone towards cleavage.

Looking at co-factor dependency, it can be clearly noticed that higher degree of cleavage was observed in liver S9 extracts in the presence of co-factor. NADPH serves as a co-factor for cytochrome P₄₅₀ isozymes, which are highly expressed in the liver. This can be considered as a further indication for liver targeting of synthesised phosphoramidates.

When stabilities in different species were compared, it was noticed that monkey and rat data were comparable with human results. However results obtained from the dog showed a different pattern, where ProTides were found to be more stable in the liver than in intestinal S9 preparations.

These results have clarified potential use of rat as a small animal model, and showed preference of monkey data over dog.

4.8.3 Stability in simulated gastric and intestinal fluids.

During oral administration, in order to be absorbed, a drug must be stable in various pHs and survive in the presence of digestive enzymes. Gastrointestinal fluids, their volume and composition can affect drug absorption, as well as presence of bile and mucosa, blood perfusion or surface and nature of epithelial membranes.²⁵ Thus, investigation of ProTide stability, especially in stomach and small intestine environment was of interest.

The pH of the fluids in the fasted-state stomach is usually in a range of 1.5-2.5. Food intake results in rapid increase of the gastric pH to values between 4-7 (depending on a content of a meal) and soon after the pH of the gastric fluids returns to the fasted-state values.²⁵

Fasted-state pH in small intestine is usually reported as ca. 6.5 and increases up to 7.5 - in the distal small intestine, but can alter between 2 and 8. After a meal, the pH in this part of intestinal tract slowly decreases, as acidic gastric content enters intestine, before returning to fasted-state values at the completion of digestion.²⁵

Stability of ProTides in simulated gastric (SGF) and intestinal fluids (SIF) was measured and evaluated by Inhibitex Inc. Reported data correspond to the stability of **4.13f**. The compound was incubated (10mg/ml PO formulation (5% DMA, 20% Solutol HS 15, 20% PEG 400, 55% 50mM sodium acetate, pH 4.0), diluted 1:10) in the appropriate medium, and percentage of recovery was measured at 15 min, 30 min, 1 h and 4 h time points at 37 °C.

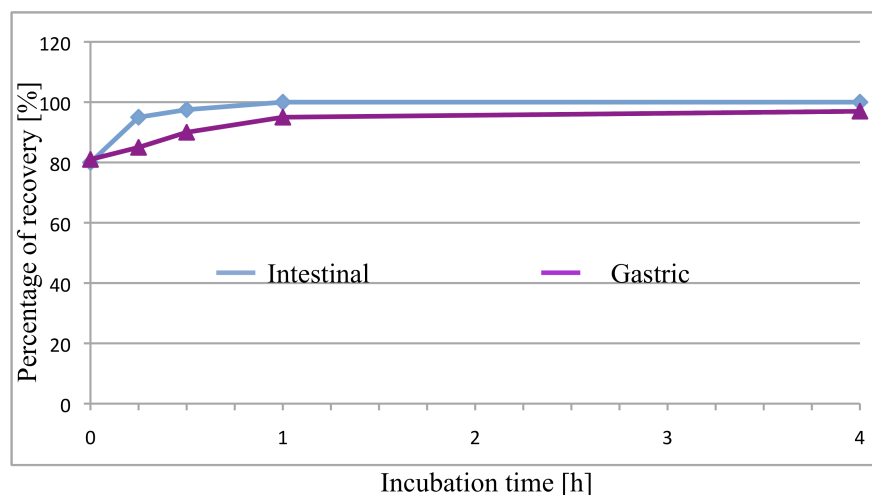


Figure 4.12. Stability of **4.13f**, in simulated intestinal and gastric fluids.

Simulated fluids used for the experiment, correspond to the fasted-state gastric conditions (pH 2, without pepsin)- for SGF, and pH 7.5 without pancreatin for SIF.

As can be seen in the Figure 4.12, phosphoramidate **4.13f** showed very good stability, with full recovery of **4.13f** within 4 hours of incubation, in both gastric and intestinal simulated fluids. This profound stability in various matrices indicates that phosphoramidates have a pH stability profile consistent with oral administration. The fact that **4.13f** was entirely stable during 4 h of incubation at pH 2 in SGF, at 37 °C, indicate, that the compound can be taken on an empty stomach and be able to pass through intact, and then undergo absorption in small intestine. Because the small intestine is the main absorption site, gastric emptying is often rate limiting step. Thus, food (especially fatty food) slows gastric emptying and consequently rate of absorption.

Stability in intestinal fluids can guarantee optimal time for the compound to be absorbed while passing through the intestinal tract before being excreted.

All these data strongly support oral administration as a route of choice for these phosphoramidates.

4.9 Intracellular levels of 2'-C-methylguanosine triphosphate – *in vitro* studies.

4.9.1 Intracellular metabolism in HCV replicon cells.

In order to determine whether synthesised ProTides can be converted intracellularly to the 2'-C-methylguanosine triphosphate (2'CMeGTP), the active species involved in the HCV NS5B inhibition, several ProTides were investigated in genotype 1b replicon cells (Inhibitex Inc.).

Replicon cells were incubated with **4.13f** and **4.13k** at concentrations that produce 50% inhibition of HCV replicon (EC_{50} = 0.05 μ M and 0.08 μ M, respectively) and 90% of inhibition (EC_{90} = 0.2 μ M and 0.4 μ M, respectively). Cells were harvested after 6 h of exposure to the ProTides, and the intracellular levels of 2'-C-methylguanosine triphosphate were measured (Figure 4.13).

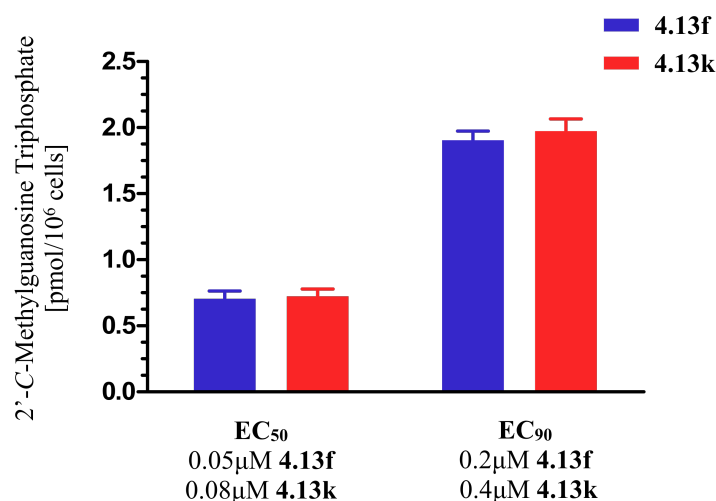


Figure 4.13. Levels of 2'CMeGTP in HCV 1b replicon system.

At 0.2 μ M **4.13f**, the concentration of 2'CMeGTP produced in replicon cells was 1.9 ± 0.42 pmol/ 1×10^6 cells. Taking in consideration liver cellularity of 1×10^8 cells per g,²⁶ the expected amount of triphosphate that would result in 90% of viral inhibition in the liver tissue, was calculated to be 190 pmol/g of liver. This value was used as a reference for further *in vivo* evaluation of triphosphate levels produced from **4.13f**. Levels of the 2'CMeGTP obtained from 0.4 μ M of **4.13k** were very similar and would correspond to the 195 pmol of triphosphate per gram of liver tissue.

4.9.2 Intracellular metabolism in primary human hepatocytes.

Further investigation of triphosphate production was carried out in primary human hepatocytes and evaluated by Inhibitex Inc. ProTides were incubated in primary human hepatocytes at an arbitrary concentration of 2 μM . Triphosphate levels were measured over a period of 6 h and for the most interesting compounds in the prolonged incubation time of 24 and 48 h. Additionally levels of 2'CMeGTP resulting from the incubation of 2'-C-methylguanosine **4.10** during 24 h and 48 h were also measured.

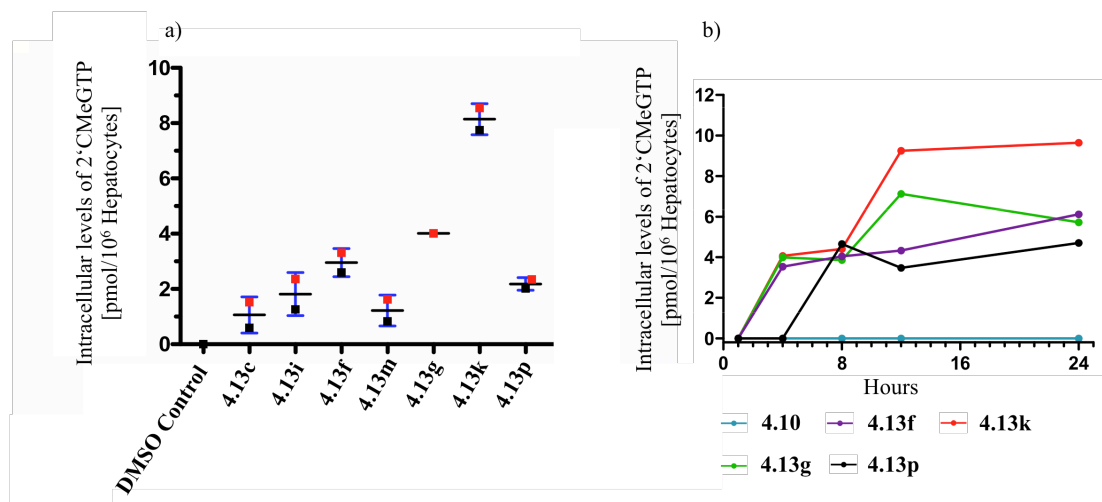


Figure 4.14. a) Levels of 2'CMeGTP produced from selected phosphoramidates in primary human hepatocyte system (6h). Black symbol = 1st analytical run; Red symbol = 2nd analytical run, b) 24h time course of 2'CMeGTP production in primary human hepatocytes.

Several structurally distinctive ProTides were chosen for the study, including L-alanine (**4.13c**, **4.13f**, **4.13g**, **4.13i** and **4.13k**) and L-valine derivatives (**4.13m** and **4.13p**), and corresponding results are shown in Figure 4.14. Incubation of all compounds resulted in formation of decent amounts of triphosphate within incubation times as short as 6 h. Looking at the L-alanine family it can be clearly seen that the highest production of 2'CMeG triphosphate was obtained from the incubation of **4.13k** (8.2 ± 0.31 pmol/1 $\times 10^6$ cells at 6 h) followed by **4.13g** (4.1 pmol/1 $\times 10^6$ cells at 6 h) and **4.13f** (2.9 ± 0.28 pmol/1 $\times 10^6$ cells at 6 h). Values for these 3 compounds were above EC₉₀ calculated in replicon assay (1.9 ± 0.42 pmol/1 $\times 10^6$ cells). L-Valines in general were found to produce less 2'CMeG-triphosphate, comparing to L-alanine phosphoramidates (**4.13i** vs. **4.13m**). These findings correlate with the ease of activation for the appropriate ProTides. Additionally levels of 5'-triphosphate

produced from the parent nucleoside **4.10** were undetectable in this particular experiment (Figure 4.14b).

Compounds **4.13f** and **4.13k** were chosen for prolonged (48 h) incubation studies (Figure 4.15). The data indicates that both phosphoramidates produce a substantial amount of 2'-C-methylguanosine triphosphate comparing with the parent nucleoside. Looking at the curve corresponding to the **4.13f**, maximum concentration (C_{\max}) of TP (42 pmol/ 10^6 cells) forms within first 12 h, and then slowly decays. For compound **4.13k**, C_{\max} has very similar value of ca. 40 pmol/ 10^6 cells, however it was achieved in much shorter time (6 h vs. 12 h). Based on these data, the half-life of intracellular 2'CMeGTP in primary human hepatocytes was calculated to be approximately 36 h (Figure 4.15). Levels of 5'-triphosphate resulted from the incubation of both **4.13f** and **4.13k** were almost 20 times higher than the concentration required to eradicate 90% of the viral load, indicating their undisputed potential as anti-HCV agents.

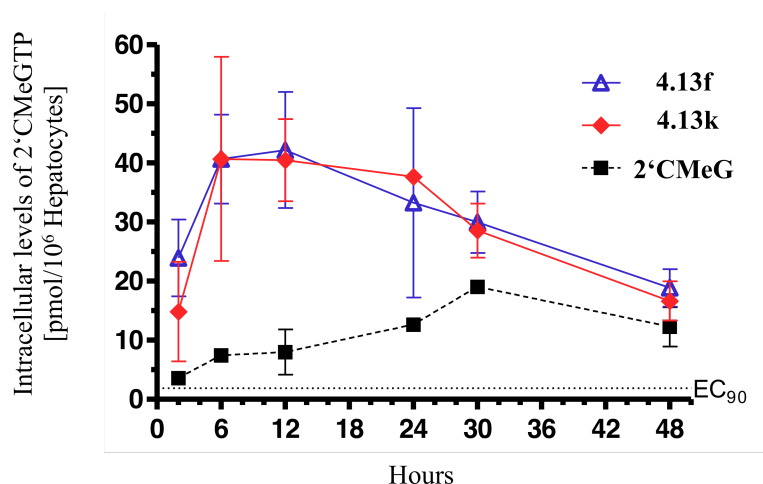


Figure 4.15. Levels of 2'CMeGTP produced from **4.13f** and **4.13k** in primary human hepatocytes system over 48 h incubation period.

On the other hand 2'CMeG was found to slowly build up triphosphate levels, with much lower and delayed C_{\max} of around 18 pmol/ 10^6 cells at 30 h. Figure 4.15 evidently shows advantage of phosphoramidate ProTides over parent nucleoside.

4.10 Intracellular levels of 2'-C-methylguanosine triphosphate – *in vivo* studies.

The ability of phosphoramidate ProTides of 2'-C-methylguanosine to deliver monophosphate inside the liver cells and subsequent formation of 2'CMeGTP after

oral administration was investigated in different animal models. All animal studies were evaluated by Inhibitex Inc.

4.10.1 Intracellular levels of 2'-C-methylguanosine triphosphate in mouse model.

A series of ProTides was evaluated in mouse model in order to evaluate their metabolism and pharmacokinetics. Mice were dosed orally at 50mg/kg (~240 mg human equivalent dose HED) in PO formulation (5% DMA, 205 Solutol HS 15, 20% PEG 400, 55% 50 mM sodium acetate, pH 4.0). Liver samples were harvested at specified time points and snap frozen. Levels of 2'-C-methylguanosine were analysed using LC-MS/MS tandem spectroscopy. At the same time generation of metabolites (mainly 2'CMG) in the plasma was measured. The main objective of this study was to find compound/s that would express high levels of liver triphosphate and at the same time low systemic nucleoside exposure. It has to be noted that the oral dose of 50mg/kg (of different compounds) is not molar equivalent dose, and therefore reported data are not exact comparisons.

For all tested phosphoramidates systemic levels of parent ProTide were not detectable and furthermore all ProTides resulted in low systemic exposure of parent nucleoside (Table 4.7).

| Cpd | C_{max} [ng/mL] | C_{last} [ng/mL] (h) | $T_{1/2}$ [h] | AUC_{0-t} [ng·h/mL] | AUC_{0-inf} [ng·h/mL] | DNAUC |
|--------------|----------------------|---------------------------|---------------|--------------------------|----------------------------|-------|
| 4.10 | 5203 | 22 (24) | 5.4 | 20390 | 20567 | 411 |
| 4.13f | 262 | 103 (6) | 5.2 | 1008 | 1920 | 38 |
| 4.13k | 1380 | 222 (6) | 1.8 | 3722 | 4269 | 85 |
| 4.13m | 504 | 130 (6) | 2.3 | 1611 | 2012 | 40 |
| 4.13p | 518 | 202 (6) | 3.5 | 1600 | 2447 | 49 |

Table 4.7. Systemic level of nucleoside resulted from 50mg/kg dose in mouse, measured in plasma samples.

All tested ProTides showed good conversion to the triphosphate in mouse liver (Figure 4.16), markedly exceeding EC_{90} values, with **4.13k** providing C_{max} (4 h) close to 11 x EC_{90} concentration (Figure 4.16).

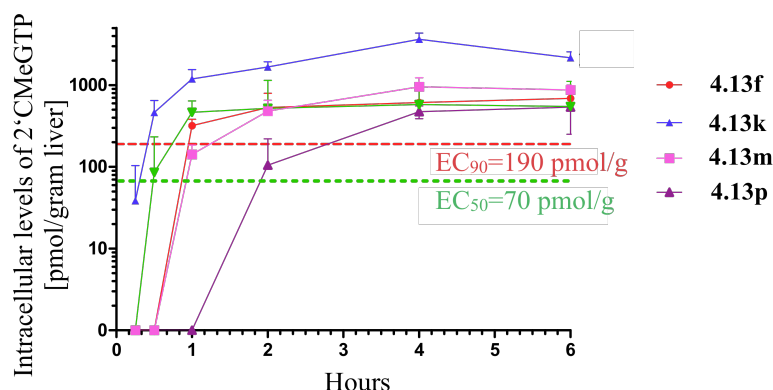


Figure 4.16. Triphosphate levels in livers of orally dosed mice.

Incubation of parent nucleoside **4.10** resulted in the highest levels of triphosphate comparing to the ProTides (Table 4.8), which is in accordance with the systemic nucleoside levels.

| Cpd | C_{\max} [ng/g] (h) | C_{last} [ng/g] (h) | AUC_{0-6} [h·ng/g] | AUC_{0-24} [h·ng/g] | DNAUC |
|--------------|--------------------------|---------------------------------|-------------------------|--------------------------|-------|
| 4.10 | 3470 (2) | 392 (24) | 16510 | 36868 | 737 |
| 4.13f | 370 (6) | 370 (6) | 1584 | | 32 |
| 4.13k | 1950 (4) | 1158 (6) | 6980 | | 140 |
| 4.13m | 512 (4) | 470 (6) | 1940 | | 97 |
| 4.13p | 290 (6) | 290 (6) | 880 | | 18 |

Table 4.8. 2'CMeGTP liver levels resulting from 50mg/kg oral dose in mouse.

This arising difference may come not only from the fact that doses were non-molar equivalents but also from excellent oral bioavailability of 2'CMeG in mouse ($F=85\%$).

Looking at ratio between the triphosphate dose normalized AUC (DNAUC) and nucleoside DNAUC it can be seen that compounds **4.13k** and **4.13m** have similar ratio to **4.10** (~ 2). The ratio obtained for **4.13f** was close to 1. With this comparison it is obvious that both ProTides **4.13k** and **4.13m**, and parent nucleoside are producing at least twice as much of triphosphate in the liver comparing to systemic nucleoside dosing. The only exemption was compound **4.13p**, which exhibited higher levels of systemic nucleoside than liver triphosphate. This finding is in contrast with the rodent stability data, where **4.13p** was found to be more stable than any of L-alanine

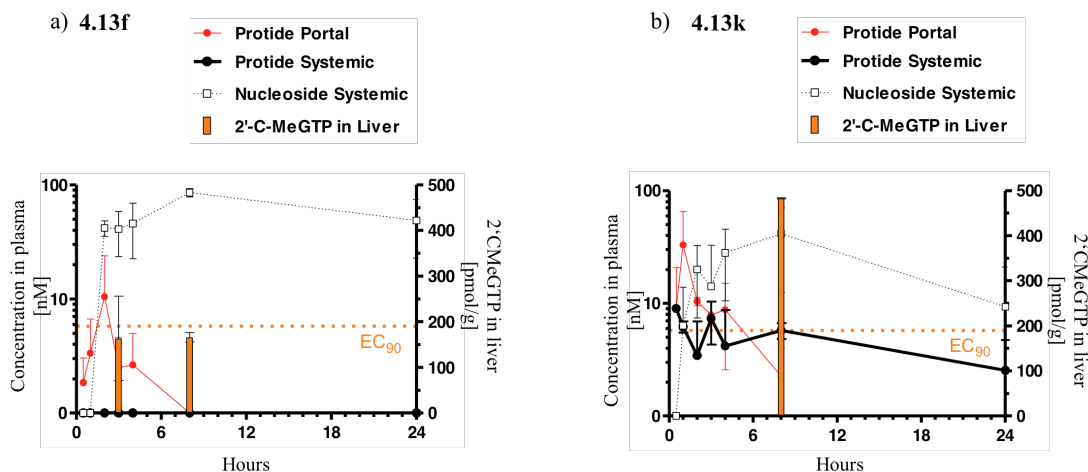
derivatives. Because the DNAUC data for ProTides are calculated using 6 h data points extrapolated out to 24 h, it is possible that the triphosphate to systemic nucleoside ratio for these compounds might be better than the one obtained for parent nucleoside **4.10**.

As already mentioned in previous sections, the mouse is considered as a non-optimal model for ProTide pharmacokinetic studies, due to the fast breakdown of phosphoramidates to the parent nucleoside, therefore several most promising compounds were investigated in the cynomolgus monkey, in which oral bioavailability of 2'CMeG is rather poor and is close to ~10%. This significant difference between oral bioavailability of 2'CMeG of these two species shows that there is a major difference in absorption and metabolism rate of 2'-C-methylguanosine and corresponding ProTides.

4.10.2 Intracellular levels of 2'-C-methylguanosine triphosphate in nonhuman primate model.

In order to determine whether phosphoramidate ProTides are able to generate 2'CMeGTP in the liver of cynomolgus monkey, four animals were orally dosed (single dose) with phosphoramidates **4.13f** and **4.13k** at 25mg/kg. Plasma samples were collected from portal and femoral veins to determine levels of the ProTide being absorbed from GI tract and systemic exposure to both, a ProTide and parent nucleoside **4.10**. Additionally levels of 2'CMeG triphosphate were measured (two post-dose time points (at 3 and 8 h) for **4.13f** and at one time point (at 8 h) for **4.13k**) from samples collected upon surgical biopsies under anaesthesia.

All results are summarised in Figure 4.17. Pharmacokinetic studies in nonhuman primates suggest efficient extraction of ProTides by the liver. In both cases phosphoramidates were detected in portal plasma samples after 30 min of dosing and continue to be present until 8 h. At this dose systemic levels of **4.13f** were not detected at any time point and **4.13k** was seen in low concentration and remained for up to 24 h post-dose. Levels of parent nucleoside started to build up 1 h post-dose for **4.13k** and 2 h for **4.13f** and correlate in a linear manner with levels of 2'CMeGTP formed in the liver.



EC₉₀: Defined as the amount of intra cellular triphosphate necessary to achieve 90% inhibition in the HCV replicon assay.

Figure 4.17. Oral pharmacokinetics in nonhuman primates.

For compound **4.13f**, liver biopsy results showed that the levels of generated triphosphate did not exceed EC₉₀ value of 190 pmol/g of liver, however concentrations of TP obtained from oral dose of **4.13k** were much greater than concentration required to eradicate 90% of the virus *in vivo*.

4.11 Conclusions.

To summarise, a series of novel 2'-C-Methylguanosine ProTides has been synthesised. In almost every case, the newly synthesised phosphoramidates were more active than the parent nucleoside, often by 10-30-fold. Structure activity relationships were extensively studied, including modification in ester, aryl and amino acid moiety. A combination of different esters and amino acids resulted in very potent compounds, suitable for further preclinical development. Plasma stability studies indicate that rodent models may not be appropriate for ProTide pharmacokinetic studies, due to the rapid breakdown of phosphoramidates to the parent 2'CMeG nucleoside. Comparing different amino acids to each other, it was noticed that L-valine phosphoramidates were among the most stable examples in mouse and rat plasma. On the other hand all tested compounds possess very good stability in dog, monkey and human plasma. Intestinal and liver S9 stability data gave an indication, that these compounds after oral administration will pass through the gastrointestinal tract and will be effectively absorbed and cleaved in the liver. Additionally, it was noted that dog model should

not be used to evaluate synthesised compounds as the ratio between intestinal to liver stability greatly differ from the one obtained for human S9 preparations. Experiments carried out in simulated gastric and intestinal fluids, confirmed acid stability of phosphoramidates and confirmed oral administration as feasible. Further development of ProTides for their ability to produce substantial levels of 2'-C-methylguanosine triphosphate, both *in vitro* and *in vivo* in different animal models, confirmed their undeniable potential.

All collected data suggest that ProTides of 2'-C-methylguanosine represent a promising class of compounds for the treatment of HCV infections.

References:

1. Merck & Co., Inc. C07d/1.521.076, **1968**.
2. Ueda T, Matsuda A, Yoshimura Y, Yakenuki K. Synthesis and biological activity of branched chain-sugar nucleosides. *Nucleosides and Nucleotides* **1989**, 8, 743-748.
3. Matsuda A, Takenuki K, Sasaki T, Ueda T. Radical deoxygenation of tert-alcohols in 1-(2-C-alkylpentofuranosyl) pyrimidines: Synthesis of (2'S)-2'-deoxy-2'C-methylcytidine, an antileukemic nucleoside. *J Med Chem* **1991**, 34, 234-239.
4. Matsuda A, Nakajima Y, Azuma A, Tanaka M, Sasaki T. 2'-C-Cyano-2'-deoxy-1-beta-D-arabinofuranosylcytosine (CNDAC): design of a potential mechanism-based DNA-strand-breaking antineoplastic nucleoside. *J Med Chem* **1991**, 34, 2917-2919.
5. Awano H, Shuto S, Baba M, Kira T, Shigeta S, Matsuda A. (2'S)-2'-deoxy-2'-C-methyl-iodouridine (SMIU) as a novel potent anti-herpes virus agent. *Bioorg Med Chem Lett* **1994**, 4, 367-370.
6. Carroll SS, Tomassini JE, Bosserman M, Getty K, Stahlhut M, Eldrup AB, Bhat B, Hall D, Simcoe AL, LaFemina R, Rytkowski CA, Wolanski B, Yang Z, Migliaccio G, De Francesco R, Kuo LC, MacCoss M, Olsen DB. Inhibition of Hepatitis C Virus RNA Replication by 2'-modified nucleoside analogues. *J Biol Chem* **2003**, 278, 11979-11984.
7. Eldrup AB, Allerson CR, Bennett CF, Bera S, Bhat B, Bhat N, Bosserman MR, Brooks J, Burlein C, Carroll SS, Cook DP, Getty KL, MacCoss M, McMasters DR, Olsen DB, Prakash TP, Prhave M, Song Q, Tomassini JE, Xia J. Structure-activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J Med Chem* **2004**, 47, 2283-2295.
8. Eldrup AB, Prhave M, Brooks J, Bhat B, Prakash TP, Song Q, Bera S, Bhat N, Dande P, Cook DP, Bennett CF, Carroll SS, Ball RG, Bosserman MR, Burlein C, Colwell LF, Fay JF, Flores OA, Getty KL, LaFemina RL, Leone J, MacCoss M, McMaster DR, Tomassini JE, Von Langen D, Wolanski B, Olsen DB. Structure-activity relationship of heterobase-modified 2'C-methyl ribonucleosides as inhibitors of hepatitis C virus RNA replication. *J Med Chem* **2004**, 47, 5284-5297.

9. Walton E, Jenkins SR, Nutt RF, Zimmerman M, Holly FW. Branched chain sugar nucleosides. A new type of biologically active nucleosides. *J Am Chem Soc* **1966**, 88, 4524-4525.
10. Li NS, Piccirilli JA. Efficient synthesis of 2'-C-Methylguanosine. *J Org Chem* **2006**, 71, 4018-4020.
11. Harry-O'kuru RE, Smith JM, Wolfe MS. Short flexible rout toward 2'-C-branched ribonucleosides. *J Org Chem* **1997**, 62, 1754-1759.
12. WO2004/052899 A2, PCT/US03/39643
13. Vorbrüggen H, Krolikiewicz K, Bennau B. *Chem Ber* **1981**, 114, 1234-1255.
14. Derudas M, Carta D, Brancale A, Vanpouille C, Lisco A, Margolis L, Balzarini J, McGuigan C. The application of phosphoramidate ProTide technology to acyclovir confers anti-HIV inhibition. *J Med Chem* **2009**, 52, 5520-5530.
15. Cahard D, McGuigan C, Balzarini J. Aryloxy phosphoramidate triesters as ProTides. *Mini-Rev Med Chem* **2004**, 4, 371-382.
16. ClogP values were calculated using ChemOffice Ultra 11.0.
17. Birkus G, Wang R, Liu X, Kutty N, MacArthur H, Cihlar T, Gibbs C, Swaminathan S, Lee W, McDermott M. Cathepsin is the major hydrolase catalyzing the intracellular hydrolysis of the antiretroviral nucleotide phosphoramidate prodrugs GS-7340 and GS-9131. *Antimicrob Agents Chemother* **2007**, 51, 543-550.
18. Hiraiwa M. Cathepsin A/protective protein: an unusual lysosomal multifunctional protein. *Cell Mol Life Sci* **1999**, 56, 894-907.
19. Sofia MJ, Furman PA, Symonds WT. 2'-F-2'-C-Methyl nucleosides and nucleotides for the treatment of hepatitis C virus: from discovery to the clinic. RSC Drug Discovery Series No. 4, Accounts in Drug Discovery: Case Studies in Medicinal Chemistry 2011, Chapter 11.
20. Saboulard D, Naesens L, Cahard D, Salgado A, Pathirana R, Velazquez S, McGuigan C, De Clercq E, Balzarini J. Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. *Mol Pharmacol* **1999**, 56, 693-704.
21. Munger JS, Shi GP, Mark EA, Chin DT, Gerard C, Chapman HA. A serine esterase released by human alveolar macrophages is closely related to liver microsomal carboxylesterase. *J Biol Chem* **1991**, 28, 18832-18838.

22. Satoh Y, Kadota Y, Oheda Y, Kuwahara J, Aikawa S, Matsuzawa F, Doi H, Aoyagi T, Sakuraba H, Itoh K. Comparative analysis of action on homologous enzymes derived from man, yeast and wheat. *J Antibiot* **2004**, 57, 316-325.
23. Mehellou Y, Valente R, Mottram H, Walsby E, Mills KI, Balzarini J, McGuigan C. Phosphoramidates of 2'- β -D-arabinouridine (AraU) as phosphate prodrugs; design, synthesis, in vitro activity and metabolism. *Bioorg Med Chem* **2010**, 18, 2439-2446.
24. McGuigan C, Cahard D, Sheeka HM, De Clercq E, Balzarini J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of a novel intracellular metabolite. *J Med Chem* **1996**, 39, 1748-1753.
25. Soderlind E, Dressman JB. Physiological factors affecting drug release and absorption in the gastrointestinal tract. Oral drug absorption: prediction and assessment. *Drugs and Pharmaceutical Sciences*. 2nd Edition, **2010**, 193, 1-20.
Wilson ZE, Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br J Clin Pharmacol* **2003**, 56, 433-440.

CHAPTER FIVE

2'-C- β -methylguanosine: new C-6 base modifications.5.1 6-C-modified 2'-C- β -methylguanosines – the rational behind the design.

Extensive studies carried out on 2'CMeG ProTides resulted in compounds like **4.13i** being ~50 times more potent than the parent nucleoside **4.10** (Figure 5.1).

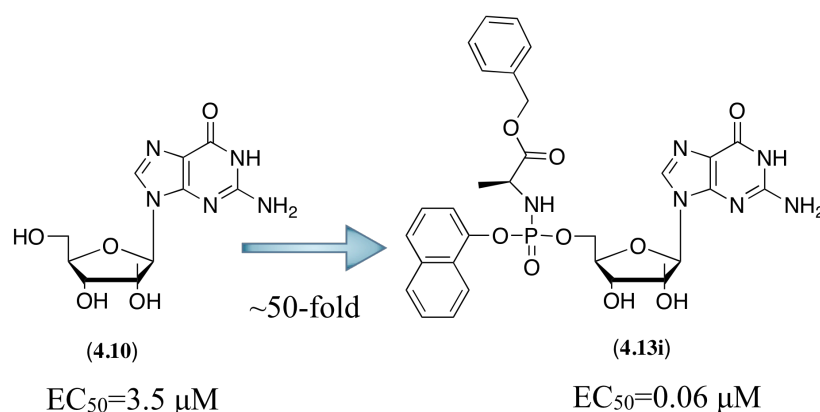


Figure 5.1. Comparison of 2'CMeG and its ProTide.

However, subsequent studies addressing plasma stability revealed that initial family of L-alanine ProTides exhibits high degree of rodent plasma instability. Further examination of different amino acid derivatives was performed in the hope of finding compounds with acceptable rodent plasma stability. This investigation led to the compounds like **4.13m** and **4.13u** bearing L-valine benzyl ester and L-isoleucine benzyl ester respectively, possessing desired stability. Nevertheless these compounds were significantly less active than their corresponding L-alanine analogue **4.13i**.

As a great variety of amino acid and ester combinations was examined, attention was focused on the nucleoside itself. It was postulated that purine base modifications could enhance the uptake and the potency of phosphoramidates and provide better pharmacokinetic profiles.

It is known that modification at C6-position may improve PK profiles. Studies carried out on guanine arabinoside (ara-G) showed that 6-O-methylation of the base unit provides a water-soluble prodrug of ara-G (Figure 5.2). Modification of the

purine base increased water solubility by 8-fold at 25 °C (pH 7.2) and 6-fold at 37 °C.¹

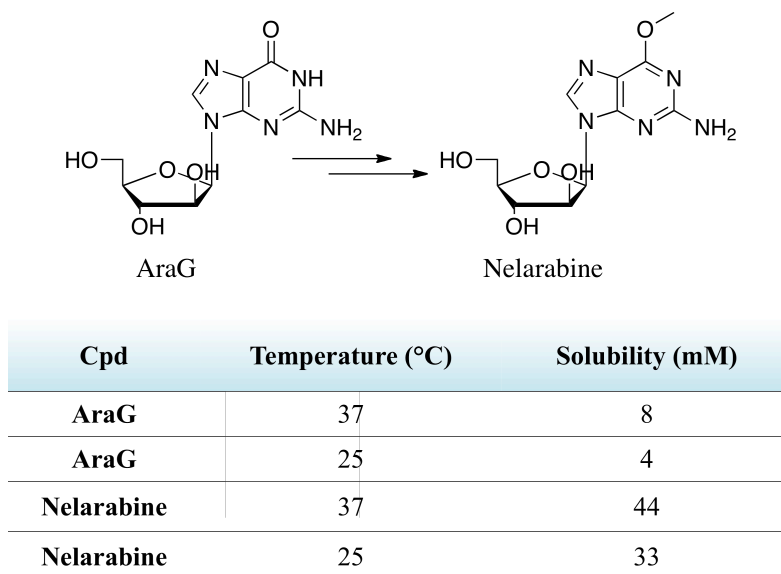


Figure 5.2. Aqueous solubility of AraG and its prodrug nelarabine in different temperatures.

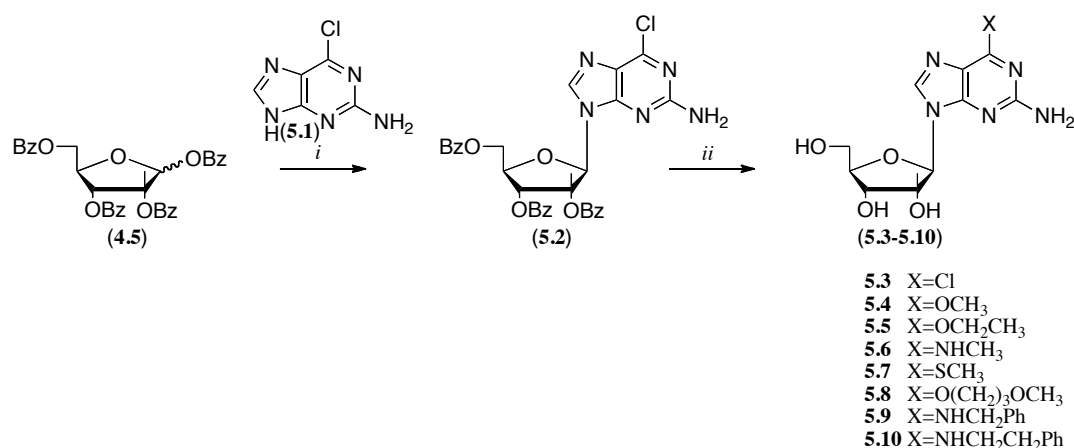
This compound is known as nelarabine – a drug used in T-cell acute lymphoblastic leukaemia. Nelarabine is metabolised by adenosine deaminase to the arabinofuranosylguanine, which is further phosphorylated to the active specie.¹

In the case of 2'-C- β -methylguanosine different substitutions in the C-6 position of the purine base were investigated, in order to increase lipophilicity of the nucleoside and thus potentially facilitate poor cellular uptake of **4.10**. It has to be noted, that these substitutions are designed to liberate 2'-C- β -methylguanosine (at nucleoside level) and 2'-C- β -methylguanosine 5'-monophosphate (at nucleotide level) after being processed by specific deaminases, and therefore act as nucleos(t)ide pro-drugs.

5.2 Synthesis of 6-C-modified 2'-C- β -methylguanosine analogues.

The synthetic route consists of the 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl- β -D-ribofuranose **4.5** synthesis² followed by the glycosylation step with 2-amino-6-chloropurine **5.1**. The coupling reaction was carried out in dry acetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and TMS-triflate at 65 °C for 4-6 hours.³ Pure compound was obtained by crystallization from methanol. Regarding the ready access to that important precursor, synthesis was repeated several times on

5-10g scale. Compound **5.2** was used as synthetic intermediate for the synthesis of the following nucleoside analogues **5.3-5.10** (Scheme 5.1).



Reagents and Conditions: *i*) DBU, TMSOTf, dry ACN, 65 °C, 4-6 h; *ii*) NH₃/MeOH, rt, 12 h, sealed tube (for **5.3**); NaOMe/MeOH, rt, 16 h (for **5.4**); NaOEt/EtOH, 50 °C, 5 h (for **5.5**); MeNH₃/MeOH/Et₃N, 85 °C, 16 h, sealed tube (for **5.6**); NaSMc/H₂O, DMF, rt, 2 h (for **5.7**); NH₃/MeOH, rt, 12 h, sealed tube, then 3-methoxy-1-propanol, NaH, THF (for **5.8**); Benzylamine, EtOH, reflux, 16 h (for **5.9**); Phenylethylamine, EtOH, reflux, 16 h, then NH₃/MeOH, rt, 16 h (for **5.10**).

Scheme 5.1. Synthesis of 6-C-modified 2'-C-β-methylguanosine analogues.

In order to obtain nucleoside analogue **5.3**, protected 2-amino-6-chloro-9-(2'-C-methyl-β-D-ribofuranosyl)purine **5.2** was suspended in methanol in a pressure tube, cooled to 0 °C and saturated with ammonia.⁴ Reaction mixture was allowed to warm to ambient temperature and was stirred for 16 h (Scheme 5.1). After that time solvents were evaporated under reduced pressure and crude mixture was purified on silica gel, using CHCl₃/MeOH (9:1) as an eluent. Pure compound was obtained as a white solid, in 90% yield.

Compound **5.4** was obtained in the reaction of **5.2** with sodium methoxide in methanol, at ambient temperature.⁴ After completion of the reaction, reaction mixture was neutralized with acetic acid, however this procedure resulted in a salt formation. As an alternative acidic form of Amberlite (H⁺) was used to neutralize the reaction mixture. Resin was added to the reaction mixture until the pH was adjusted to the value of 7-8. After that, resin was filtered off and solvent was removed in vacuum. After purification pure **5.4** was obtained as a white solid. The excess of sodium methoxide used in the reaction allowed final deprotection of the nucleoside derivative (Scheme 5.1). Conversion of **5.2** to **5.4** was straightforward and high yielding – 78%.

Synthesis of the 2-amino-6-ethoxy-9-(2'-*C*-methyl- β -D-ribofuranosyl) **5.5** was performed using similar procedure as described above, using sodium ethoxide in ethanol, at 50 °C for 16 h (Scheme 5.1).⁴ After purification the compound was obtained as a white solid in 84% yield.

6-Methylamino analogue **5.6** was synthesised in the reaction of **5.2** with 25% solution of methylamine in methanol, in the presence of triethylamine.^{4,5} Reaction was performed in a sealed tube, at 85 °C for 16 hours (Scheme 5.1). Displacement of the chlorine and deprotection were performed in one step, in 90% yield.

Replacement of the oxygen atom at the 6-position of guanine base by a sulfur atom, to produce clinically useful agents is known in the literature.⁶ Therefore, it was decided to investigate the influence of thiomethoxy substitution at the 6-position. Sodium methanethiolate (15% in H₂O) in dimethylformamide (DMF) was used to convert 2-amino-6-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine to **5.7** (Scheme 5.1). After being stirred at ambient temperature for 2h, the mixture was diluted with water and extracted 3 times with ethyl acetate.⁷ Column chromatography provided pure **5.7**, in 86% yield. As in the previous reactions, deprotection of the benzoyl-protecting groups took place under reaction conditions.

In the first attempt towards **5.8**, 3-methoxy-1-propoxide was formed in situ from 3-methoxy-1-propanol and sodium hydride (NaH) in dry THF, at 0 °C.⁸ The reaction was allowed to stir for 30 min at ambient temperature before addition of 2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine. The reaction mixture was stirred for 16 hours at ambient temperature. After that time, the reaction was quenched by the addition of Amberlite (H⁺). The resin was filtered off and solvents were removed under reduced pressure. Crude mixture was purified on silica gel, using CHCl₃/MeOH (93:7) as an eluent. Analysis of isolated fractions revealed that during the displacement reaction partial deprotection of the nucleoside analogue took place. Fractions containing partially deprotected nucleoside were combined and submitted for the ammonolysis step. After completion of the reaction it was found that, 3-methoxy-1-propoxy substitution was not stable under the conditions of the deprotection reaction, and was cleaved off. To avoid unwanted side-products, it was decided that the displacement of the chlorine atom with 3-methoxy-1-propoxide will be performed on already deprotected 2-amino-6-chloro-9-(2'-*C*-methyl- β -D-

ribofuranosyl)purine nucleoside **5.3** instead of **5.2** (Scheme 5.1). After completion of the reaction and purification, **5.8** was successfully isolated in 50% yield.

Treatment of **5.2** with benzylamine or phenylethylamine in refluxing dry ethanol, resulted in formation of **5.9** and **5.10**, respectively (Scheme 5.1).⁹ In case of **5.10** additional deprotection step was required as phenylethylamine was not basic enough to remove benzoyl-protecting groups. Both nucleosides were obtained in excellent yields >90%.

5.2.1 Biological evaluation of 6-*C*-modified 2'-*C*- β -methylguanosine analogues.

All newly synthesised compounds were assayed for their ability to inhibit HCV RNA replication in subgenomic replicon cells by Inhibitex Inc. The potency and toxicity of these compounds is summarised in Table 5.1.

| Cpd | X | ClogP | EC ₅₀ (μ M) | CC ₅₀ (μ M) |
|-------------|---|-------|-----------------------------|-----------------------------|
| 4.10 | OH | -2.6 | 3.5 | >100 |
| 5.3 | Cl | -0.9 | 8.5 | >100 |
| 5.4 | OCH ₃ | -0.6 | 4.6 | >100 |
| 5.5 | OCH ₂ CH ₃ | -0.1 | 8.8 | >100 |
| 5.6 | NHCH ₃ | -0.7 | 11 | >100 |
| 5.7 | SCH ₃ | -0.4 | 13 | >100 |
| 5.8 | O(CH ₂) ₃ OCH ₃ | -0.4 | 12 | >100 |
| 5.9 | NHCH ₂ Ph | 0.7 | 30 | >100 |
| 5.10 | NHCH ₂ CH ₂ Ph | 1.4 | 24 | >100 |

ClogP values calculated using ChemOffice ultra 11.0.; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 5.1. Anti-HCV activity and calculated lipophilicity of C-6 substituted 2'-*C*- β -methylguanosine analogues.

As can be seen in Table 5.1, all C6-substituted nucleoside analogues exhibit improved ClogP values, comparing to the 2'-*C*- β -methylguanosine **4.10**, with compounds **5.9** and **5.10** possessing the most enhanced lipophilicity. Not much difference in ClogP values was seen between 6-Cl, -NHCH₃ and OCH₃. Similar values were also predicted for compounds **5.5**, **5.7** and **5.8**. When anti-HCV activity was measured, it was found that the EC₅₀ values do not correlate with compounds improved ClogP. In general, all compounds were active at μ M range, however none

of the newly synthesised analogues was more potent than **4.10**. Small substituents e.g. 6-methoxy **5.4** retain the activity, while the presence of larger groups at C-6 resulted in significant decrease in activity, by 8-10-fold (**5.10** and **5.9**).

5.2.3 Adenosine deaminase assay on 6-C-modified 2'-C- β -methylguanosine analogues.

When this family of C6-modified nucleosides was designed, it was kept in mind that they will act as pro-drugs of 2'-C- β -methylguanosine. In order to investigate whether C6-substituted analogues can serve as substrates for adenosine deaminase, an enzymatic assay in the presence of adenosine deaminase from calf intestinal mucosa was performed.

Adenosine deaminase (ADA, EC 3.5.4.4) and adenylate deaminase (5'-adenylic acid deaminase, AMPDA, EC 3.5.4.6) are enzymes that catalyze the deamination reaction of adenosine and adenosine 5'-monophosphate to the corresponding inosine derivatives.¹⁰ ADA is able to catalyze the deamination of structurally distinctive purine nucleosides, provided that 5'-hydroxyl group is present and free. AMPDA was found to be less specific than ADA, with a broader spectrum of substrate specificity.¹¹

44 μ M Solutions of the appropriate C-6 substituted nucleosides in phosphate buffer (pH 7.4) were prepared. 1 mL of solution was placed in a cuvet and a blank spectrum was recorded using a UV spectrophotometer (red curve, Figure 5.3). After that 30 μ L of ADA solution were added and spectra were recorded in 1-minute intervals. As can be seen in Figure 5.3, starting material **5.4** with maximum absorbance at $\lambda_{\text{max}} = 280$ nm, was easily processed to form a new species (blue curve) with $\lambda_{\text{max}} = 253$ nm. It was found that this new species corresponds to the 2'-C-methylguanosine nucleoside as expected. Full conversion of **5.4** to **4.10** was accomplished within 30 min.

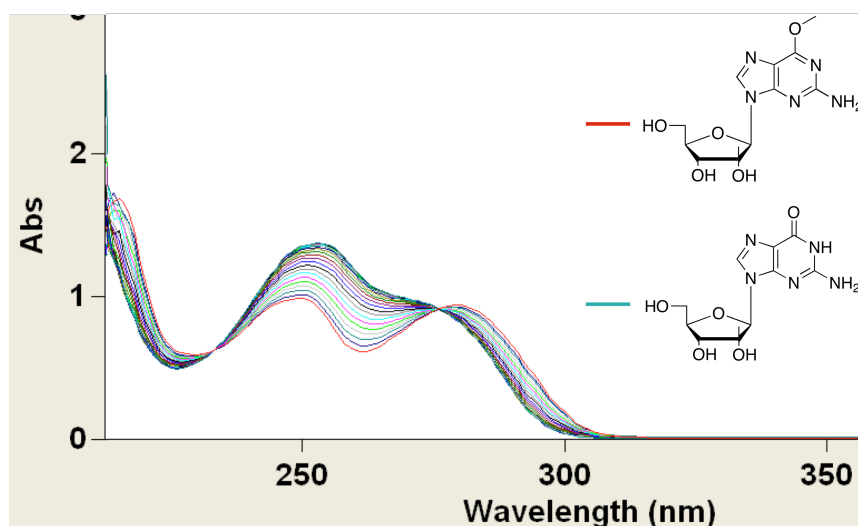


Figure 5.3. Spectral change during ADA-mediated hydrolytic removal of alkyl group from 5.4.

Similarly compound 5.5 was investigated for its ability to serve as a substrate for the adenosine deaminase mediated cleavage (Figure 5.4).

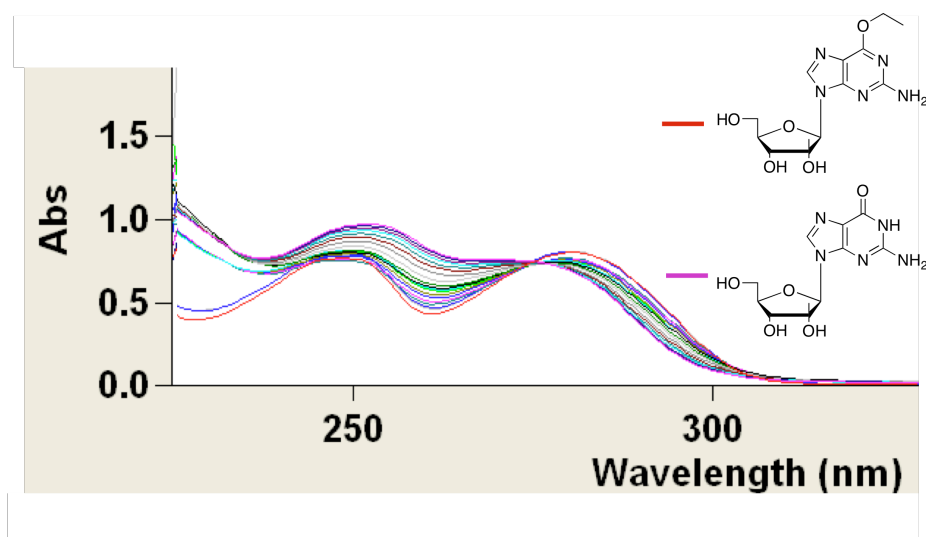


Figure 5.4. Spectral change during ADA-mediated hydrolytic removal of alkyl group from 5.5.

Nucleoside analogue 5.5 was found to be suitable substrate for the enzyme, however the conversion rate to 4.10 was much lower than in the case of 5.4. Full conversion was achieved after further addition of an enzyme (20 μ L) and within 8 hours (Figure 5.4).

Compounds 5.6 and 5.7 were also tested in the adenosine deaminase assay, but neither of them served as substrates for the enzyme (Figure 5.5).

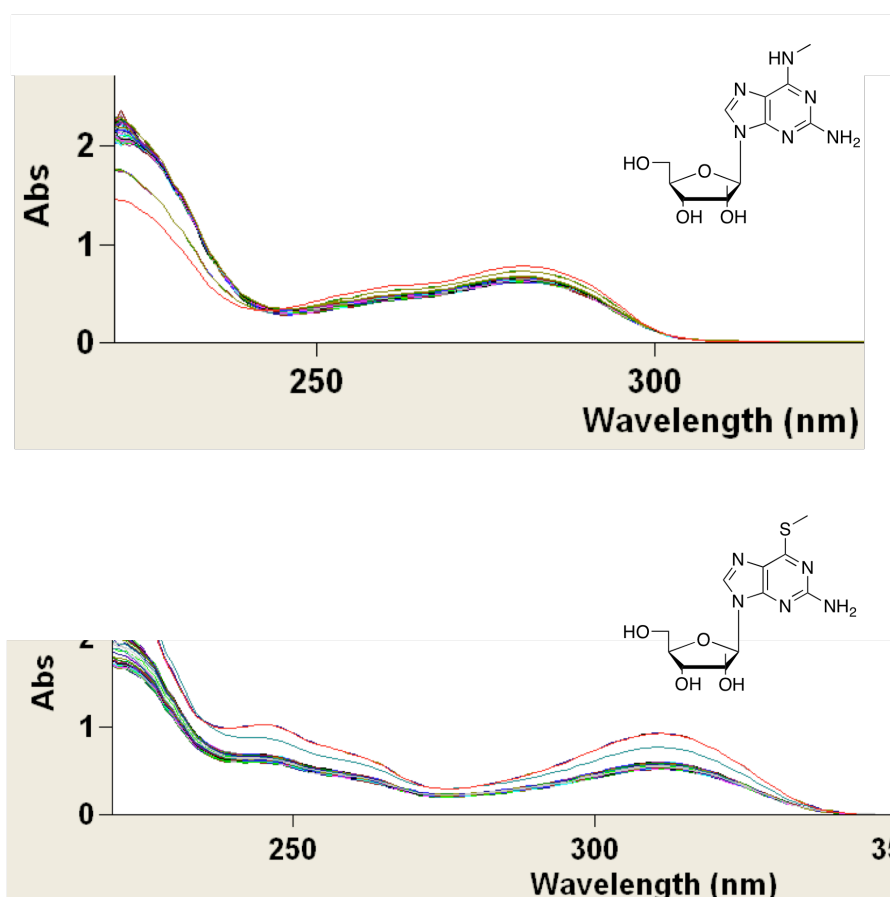


Figure 5.5. Spectral change during ADA-mediated hydrolytic removal of alkyl group from **5.6** and **5.7**.

It is not clear whether the corresponding 5'-monophosphate forms of these nucleosides could serve as substrates for adenylic acid deaminase or other hydrolytic enzymes. AMPDA is not commercially available and therefore more appropriate studies have not been conducted. Based on the literature, adenosine deaminase-like protein 1 (ADAL1) may be an alternative enzyme responsible for the hydrolytic cleavage of C-6 substituted purine 5'-monophosphates.¹² Studies carried out by Murakami *et al* show that the active site of the enzyme could accommodate modest size lipophilic N-6 substitutions of the purine analogues. According to these findings ADAL1 could be responsible for the deamination of the 5'-monophosphate of **5.6**. Furthermore 6-thiomethyl purine analogues similar to **5.7** were found to be slowly processed by the enzyme. The difference seen in conversion rate can presumably arise from the electronic influence of the thioether on C-6 carbon, reducing its electrophilicity and therefore make it less susceptible for the nucleophilic attack by the water molecule.

Compounds **5.9** and **5.10** bearing benzylamino- and phenylethylamino- substituents in C-6 position were not deaminated in ADA assays (data not shown) possibly because the side groups are too large to be accommodated in the active site of the enzyme.

To further support the requirement of adenosine deaminase mediated activation of C-6-modified nucleosides, replicon activity assay in the presence of 2 μ M erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) - specific inhibitor of ADA was performed on 6-*O*-methyl-2'-*C*-methylguanosine **5.4** (Figure 5.6) and evaluated at Inhibitex Inc.

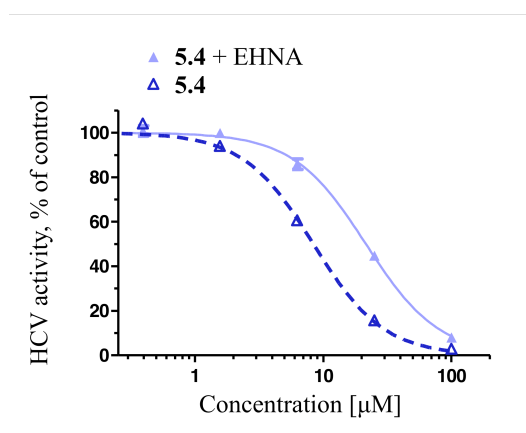


Figure 5.6. Effect of EHNA on anti-HCV activity of nucleoside **5.4**.

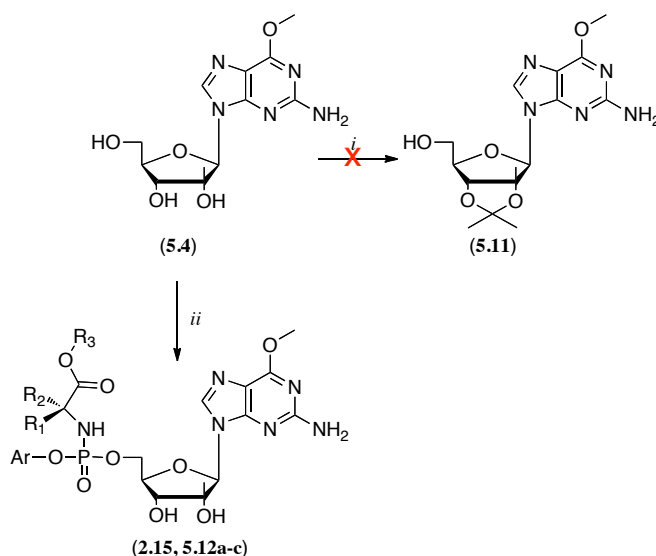
Data with ADA-specific inhibitor EHNA (light blue curve) clearly suggest that the hydrolysis of substitution at C-6 carbon is required for the compound to obtain its antiviral activity (dark blue curve). When replicon cells were treated with combination of **5.4** and 2 μ M EHNA, no or little antiviral effect was observed up to 10 μ M concentration (Figure 5.6). Under the same conditions **5.4** alone exhibited more than 50% inhibition.

This study provided proof of concept and revealed the potential of O6 and/or N6 2-aminopurine nucleosides as anti-HCV agents.

The aryloxyphosphoramidate approach was applied to **5.3-5.10** in order to investigate whether the delivery of their pre-formed and masked monophosphate can enhance antiviral properties of this family of 2'-*C*-methylguanosine analogues.

5.3 Synthesis of 6-C-modified 2'-C- β -methylguanosine ProTides.

In the first instance, the phosphoramidate approach was applied to the 6-methoxy analogue **5.4** (Scheme 5.4). As in the case of 2'-C- β -methylguanosine **4.10**, 2',3'-protecting procedures were investigated. Several different approaches to introduce isopropylidene protection were not successful and the compound proved to be unstable in acidic conditions. It was decided to use NMI coupling procedure directly on the free nucleoside according to the primary hydroxyl group specificity of this reagent.¹³



Reagents and Conditions: i) HClO_4 , acetone, rt, overnight; ii) a) appropriate phosphorochloridate, NMI, dry THF, rt, overnight.

Scheme 5.2 General synthesis for 2-amino-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine phosphoramidates using NMI method.

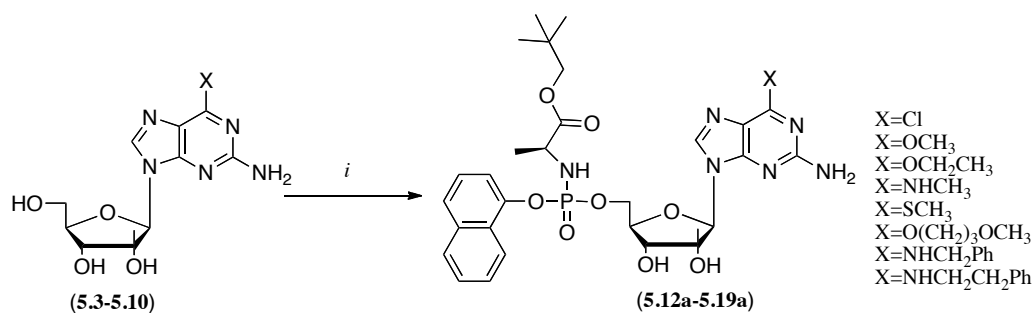
A small series of 5'-phosphoramidates (**2.15**, **5.12a** – **5.12c**) was prepared in moderate yield 11-14% (Table 5.2).

| Cpd | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | yield % |
|--------------|----------------|-----------------|----------------|-------|------------------------|-------|---------|
| 2.15 | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3.1 | 11 |
| 5.12a | α -Naph | CH ₃ | H | L-Ala | cyclopentyl | 3.9 | 14 |
| 5.12b | α -Naph | CH ₃ | H | L-Ala | cyclohexyl | 4.4 | 14 |
| 5.12c | α -Naph | CH ₃ | H | L-Ala | benzyl | 4.5 | 14 |

AA: amino acid; ClogP values calculated using ChemOffice ultra 11.0.

Table 5.2. Summary of 2-amino-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine ProTides, their calculated lipophilicity and reaction yields.

At this point it was decided to investigate other coupling conditions in an attempt to improve coupling yields. The *t*BuMgCl coupling method was chosen and applied to 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine in order to obtain **2.15**. Surprisingly during the reaction course, the formation of 3' and/or 3',5' disubstituted regioisomeric mixtures was not observed. We hypothesised that methyl substitution in 2'β-face position may have an influence, causing steric hindrance and/or changing sugar conformation, and therefore enabling reaction to proceed in the 3' position. This unexpected result gave us the opportunity to use Grignard reagent-mediated coupling in order to complete the second series of C6-modified phosphoramidates using previously synthesised nucleoside analogues **5.3** – **5.10**, with higher yields and easier purification step (Scheme 5.3, Table 5.3). All compounds were prepared as α-naphthyl L-alanine 2,2-dimethylpropyl derivatives.



Reagents and Conditions: i) Naph-L-alanine-2,2-dimethylpropyl phosphorochloridate, *t*BuMgCl, dry THF, rt, overnight.

Scheme 5.3. General procedure for the synthesis of C-6 modified nucleosides ProTides using *t*BuMgCl.

| Cpd | X | ClogP | yield % |
|--------------|---|-------|---------|
| 2.15 | OCH ₃ | 3.3 | 27 |
| 5.13a | Cl | 3.0 | 24 |
| 5.14a | OCH ₂ CH ₃ | 3.8 | 28 |
| 5.15a | NHCH ₃ | 3.2 | 17 |
| 5.16a | SCH ₃ | 3.5 | 19 |
| 5.17a | O(CH ₂) ₃ OCH ₃ | 3.2 | 25 |
| 5.18a | NHCH ₂ Ph | 4.7 | 11 |
| 5.19a | NHCH ₂ CH ₂ Ph | 5.3 | 24 |

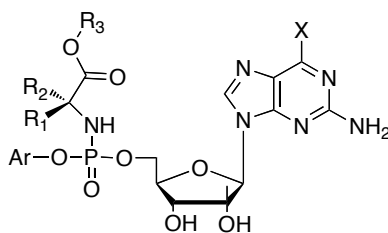
Table 5.3. Summary of synthesised ProTides of 6-modified-2'-*C*-methylguanosine, their calculated lipophilicity and isolated yields.

All newly synthesised compounds were isolated as roughly equimolar mixtures of diastereoisomers at the phosphorus centre, evidenced by ^{31}P NMR and HPLC. The first two series of C-6 modified nucleoside analogues were evaluated in *in vitro* assay, as inhibitors of HCV replication by Inhibitex Inc.

5.4 Biological evaluation of first series of 6-C-modified 2'-C- β -methylguanosine ProTides.

The first set of the new phosphoramidates was evaluated in replicon assay against hepatitis C virus, with data being shown in Table 5.4, along with comparator data of parent nucleosides **5.3-5.10**.

As noted in Table 5.4, all of the novel L-alanine phosphoramidates were active at submicromolar concentrations in the replicon assay, while all parent nucleoside were only moderately active. In every case application of ProTide technology resulted in significant increase of activity up to ca. 1000-fold (**5.14a** vs **5.5**). In particular the most active phosphoramidates were those bearing -Cl, -OMe and -OEt substitutions in C-6 position (**5.13a**, **2.15**, **5.12a-c** and **5.14a**, respectively). Some of these compounds were active at 10 nM levels, making them amongst the most potent nucleotides vs. HCV at the time. **2.15** has later emerge as the clinical candidate **INX-08189**. ProTides **5.15a-5.17a** were equipotent, exhibiting EC_{50} values of about 0.04 μM and more than 300-fold improvement comparing to the respective parent nucleosides. Phosphoramidates bearing the largest substitutions in C6-position e.g. **5.18a** and **5.19a**, lost some activity comparing to **2.15** or **5.14a**. This difference can arise most probably due to the size restriction at C6, nevertheless these ProTides were over 30-fold more potent than the parent nucleoside analogues **5.9** or **5.10**, respectively. Comparing different esters in the 6-O-methyl-2'-C- β -methylguanosine L-alanine series (entries **2.15**, **5.12a-c**, Table 5.4) it can be clearly seen, that within this small family of ProTides, not much of a difference in anti-HCV activity can be seen between the compounds bearing aliphatic or aromatic esters **5.12b** vs. **5.12c**.



| Cpd | X | Ar | R1 | R2 | AA | R3 | EC ₅₀ (μM) | CC ₅₀ (μM) |
|-------|---|--------|-----------------|----|-------|--------------------|--------------------------|--------------------------|
| 5.4 | OCH ₃ | - | - | - | - | - | 4.6 | >100 |
| 2.15 | OCH ₃ | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.01 | 7 |
| 5.12a | OCH ₃ | α-Naph | CH ₃ | H | L-Ala | cyclopentyl | 0.03 | 9 |
| 5.12b | OCH ₃ | α-Naph | CH ₃ | H | L-Ala | cyclohexyl | 0.03 | 6 |
| 5.12c | OCH ₃ | α-Naph | CH ₃ | H | L-Ala | benzyl | 0.03 | 12 |
| 5.3 | Cl | - | - | - | - | - | 8.5 | >100 |
| 5.13a | Cl | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.012 | 16 |
| 5.5 | OCH ₂ CH ₃ | - | - | - | - | - | 8.8 | >100 |
| 5.14a | OCH ₂ CH ₃ | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.01 | 10 |
| 5.6 | NHCH ₃ | - | - | - | - | - | 11 | >100 |
| 5.15a | NHCH ₃ | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.035 | 38 |
| 5.7 | SCH ₃ | - | - | - | - | - | 13 | >100 |
| 5.16a | SCH ₃ | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.037 | 41 |
| 5.8 | O(CH ₂) ₃ OCH ₃ | - | - | - | - | - | 12 | >100 |
| 5.17a | O(CH ₂) ₃ OCH ₃ | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.043 | 31 |
| 5.9 | NHCH ₂ Ph | - | - | - | - | - | 30 | >100 |
| 5.18a | NHCH ₂ Ph | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.79 | 15 |
| 5.10 | NHCH ₂ CH ₂ Ph | - | - | - | - | - | 24 | >100 |
| 5.19a | NHCH ₂ CH ₂ Ph | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 0.27 | 42 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 5.4. Anti-HCV activity of 6-modified 2'-CMeG ProTides.

All synthesised phosphoramidates exhibited cytotoxicity at μM concentrations, however taking in consideration their high potency, the values of the therapeutic indexes were in the range of a 1000. In general, the most toxic compounds were also the most potent suggesting perhaps that toxicity was arising from the active 5'-nucleoside triphosphate form or a precursor.

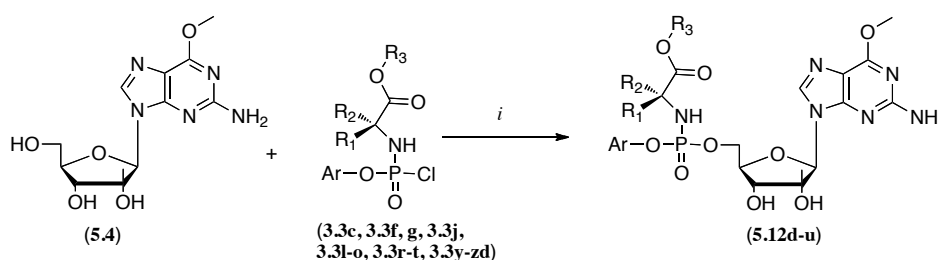
Given the very good activity of 2-amino-6-methoxy-9-(2'-C-methyl-β-D-ribofuranosyl) purine and 2-amino-6-ethoxy-9-(2'-C-methyl-β-D-ribofuranosyl) purine phosphoramidates, it was decided to further investigate these two families of ProTides.

5.5 SAR studies of 6-*O*-methyl and 6-*O*-ethyl-2'-*C*- β -methylguanosine ProTides.

Following the *t*BuMgCl coupling procedure described in the previous section of this Chapter, two series of phosphoramidates were synthesised in order to complete the 6OMe and 6OEt2'*C*MeG families. ProTides were varied at amino acid, ester and aryl moieties.

5.5.1 Synthesis and biological evaluation of 6-*O*-methyl-2'-*C*- β -methylguanosine phosphoramidates.

In the first instance 2-amino-6-methoxy-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine phosphoramidates were synthesised and biologically evaluated.



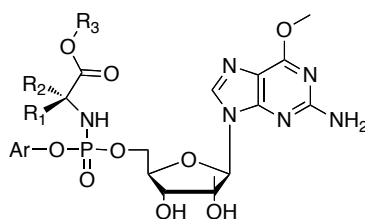
Reagents and Conditions: i) appropriate phosphorochloridate, *t*BuMgCl, dry THF, rt, overnight.

Scheme 5.4. General method for the synthesis of aryloxyphosphoramidates of 6-*O*-methyl-2'-*C*-methylguanosine using *t*BuMgCl.

All synthesised ProTides (Scheme 5.4) were routinely isolated as roughly 1:1 mixtures of phosphate diastereoisomers as evidenced by HPLC and ³¹P NMR signal splitting. The only exception was compound **5.12f**, which was isolated as a mixture of four diastereoisomers, due to the additional variable chiral centre in the ester unit. The variation of different amino acids, esters and aryl moieties lead to the compounds with a ClogP values between 1.1 and 4.6 (Table 5.5).

Compounds were evaluated for their inhibitory activity in *in vitro* replicon assay and the corresponding results are reported in Table 5.5. All synthesised phosphoramidates exhibited improved EC₅₀ values compared to the parent nucleoside **5.4**. In the L-alanine series, entries **5.12d-k**, lengthening, branching and aromatization of the ester moiety lead to agents with desired submicromolar activity. The most active derivatives were bearing 2,2-dimethylpropyl, propyl, 3,3-dimethyl-1-butyl and

S-phenylethyl esters (**2.15**, **5.12d**, **5.12e** and **5.12h**, respectively, Figure 5.7) showing 150 to 450-fold enhancement in inhibition of HCV replication.



| Cpd | Ar | R1 | R2 | AA | R3 | ClogP | EC ₅₀ (μM) | CC ₅₀ (μM) |
|--------------|-------------------------------|--|-------------------------------|-------|-----------------------|-------|-----------------------|-----------------------|
| 5.4 | - | - | - | - | - | -0.6 | 4.6 | >100 |
| 2.15 | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3.3 | 0.01 | 7 |
| 5.12d | α-Naph | CH ₃ | H | L-Ala | <i>n</i> -propyl | 2.0 | 0.03 | 13 |
| 5.12e | α-Naph | CH ₃ | H | L-Ala | 3,3-dimethyl-1-butyl | 3.8 | 0.01 | 7.6 |
| 5.12f | α-Naph | CH ₃ | H | L-Ala | 3,3-dimethyl-2-butyl | 3.6 | 0.17 | NA |
| 5.12g | α-Naph | CH ₃ | H | L-Ala | tetrahydropyranyl | 1.1 | 0.17 | >130 |
| 5.12h | α-Naph | CH ₃ | H | L-Ala | <i>S</i> -phenylethyl | 3.5 | 0.015 | 14 |
| 5.12i | α-Naph | CH ₃ | H | L-Ala | 2-indanyl | 3.4 | 0.4 | 45 |
| 5.12j | Ph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 2.1 | 0.05 | 25 |
| 5.12k | <i>p</i> -NO ₂ -Ph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 2.0 | 0.38 | >100 |
| 5.12l | α-Naph | CH(CH ₃) ₂ | H | L-Val | methyl | 2.4 | 0.36 | 22 |
| 5.12m | α-Naph | CH(CH ₃) ₂ | H | L-Val | 2,2-dimethylpropyl | 4.2 | 0.16 | 29 |
| 5.12n | α-Naph | CH(CH ₃) ₂ | H | L-Val | cyclohexyl | 4.4 | 0.4 | 29 |
| 5.12o | α-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.1 | 0.06 | 56 |
| 5.12p | α-Naph | CH(CH ₂) ₂ SCH ₃ | H | L-Met | 2,2-dimethylpropyl | 3.4 | 0.06 | 28 |
| 5.12q | α-Naph | CH(CH ₂) ₂ SCH ₃ | H | L-Met | benzyl | 3.3 | 0.23 | NA |
| 5.12r | α-Naph | CH(CH ₂) ₂ SCH ₃ | H | L-Met | <i>S</i> -phenylethyl | 3.6 | 0.32 | 30 |
| 5.12s | α-Naph | H | C ₆ H ₅ | D-PhG | <i>n</i> -propyl | 3.7 | 2.5 | 26 |
| 5.12t | α-Naph | H | C ₆ H ₅ | D-PhG | cyclohexyl | 4.6 | 2.7 | NA |
| 5.12u | α-Naph | H | C ₆ H ₅ | D-PhG | benzyl | 4.3 | 1.23 | 50 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 5.5. Summary of the anti-HCV activity of 6OMe2'CMeG ProTides and their calculated ClogP values.

The least active compound amongst the L-alanine analogues were **5.12f**, **5.12g** and **5.12i**. The explanation for the relative loss of activity for these compounds may be different in each case. For example in the case of **5.12g**, the tetrahydropyranyl L-alanine ProTide, the value of calculated lipophilicity (ClogP = 1.1) is somewhat lower

than that considered as an optimal for passive cellular uptake, and thus perhaps resulting in a decrease in activity comparing to **2.15** or **5.12e**. One important thing that has to be mentioned is that this particular analogue exhibited no cytotoxicity in the cell-based assay, and possesses the best therapeutic index (>700). The bulkiness and relative stability of 2-indanyl ester (which in some extent may be similar to the *t*-butyl ester) can be responsible for the lack of efficient activation of this particular phosphoramidate and as a result, reduction of potency of **5.12i** is observed. In the case of **5.12f**, the explanation might be that additional branching of the ester unit may give some steric hindrance and thus, processing to the corresponding 5'-monophosphate is less effective.

Looking at the relationship between compounds lipophilicity and *in vitro* anti-HCV activity, it can be noticed that compounds with ClogP below 2 and above 4 had lower potency than those with ClogP between 2 and 4 (Figure 5.7).

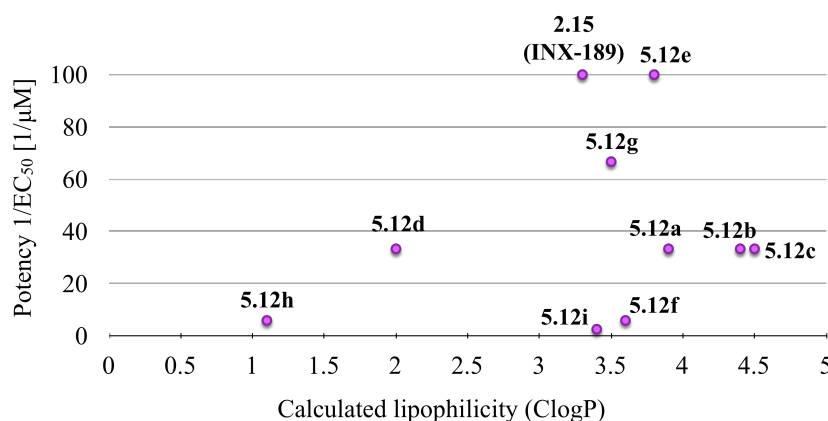


Figure 5.7. Potency ($1/EC_{50}$) and calculated lipophilicity (ClogP) data plot of synthesised phosphoramidates **2.15**, **5.12a-i**.

This range might be considered as an optimal for passive cellular uptake and therefore result in enhanced antiviral activity. The only exception were compounds **5.12f** and **5.12i**, which had ClogP in the same range, however the EC_{50} values (0.17 and 0.4 μ M, respectively) were somewhat lower. This difference may arise, as already mentioned, from the relative stability of ester groups present in the structure of these phosphoramidates.

When we compare ProTides bearing the same amino acid ester (L-Ala-2,2-dimethylpropyl) but different aryl moieties, it can be noticed that the aryl unit has an impact on the potency of synthesised compounds.

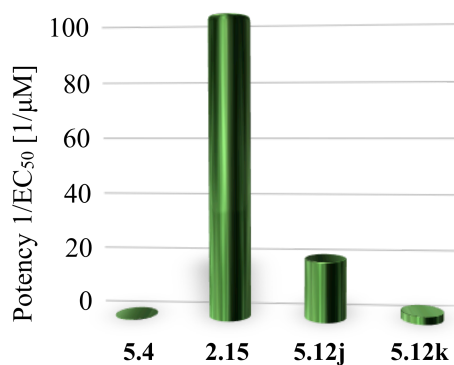


Figure 5.8. Potency ($1/EC_{50}$) data plot of L-alanine ProTides bearing different aryl moieties.

Replacement of α -naphthyl unit (**2.15**, $EC_{50} = 0.01 \mu M$) by phenyl- (**5.12j**, $EC_{50} = 0.05 \mu M$) or *p*-NO₂-phenyl- (**5.12k**, $EC_{50} = 0.38 \mu M$) resulted in 5- to ~40-fold decrease in antiviral activity seen in replicon based assay (Figure 5.8). In this set of aryl units, *p*-NO₂-phenol is recognized as the best leaving group, however the compound with this particular substituted phenol was found to be the least active within the L-Ala-2,2-dimethylpropyl family (Table 5.5, Figure 5.8). It may suggest that the compound **5.12k** is liberating phosphoramidate monoester prior entering the cell due to the very good leaving group ability of *p*-NO₂-phenol and therefore observed antiviral activity is lower.

Beside the ester and aryl moiety variation, several analogues with differed amino acids were prepared, varying the amino acid unit from L-alanine **2.15**, **5.12a-k**, to L-valine **5.12l-o**, L-methionine **5.12p-r** and unnatural amino acid D-phenylglycine **5.12s-u**. All synthesised compounds possess improved antiviral activities comparing to the parent nucleoside **5.4**, with the exemption of compounds bearing D-phenylglycine as amino acid, which did not exhibit improved potency. These results are consistent with the previous findings (see Chapter 4), and confirm that the unnatural amino acids may not lead to good substrates for carboxypeptidase-mediated activation. β -Branched amino acids like L-valine were less active than the corresponding L-alanine derivatives but still kept good antiviral activity, in sub- μM range. The most active compound across L-valine family was **5.12o** ($EC_{50} = 0.06 \mu M$), bearing a benzyl ester (Figure 5.9). This particular valine derivative was the most active L-valine ProTide synthesised so far.

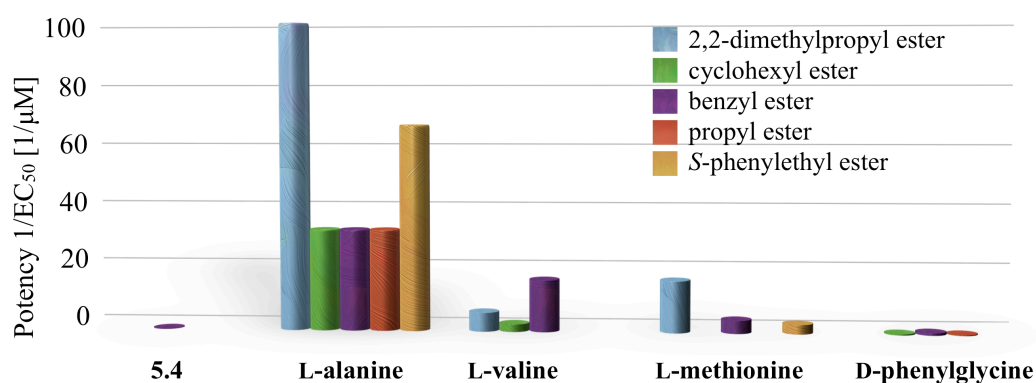


Figure 5.9. Potency ($1/EC_{50}$) data plot of various amino acid esters ProTides of 6OMe2'CMcG.

Longer amino acids like L-methionine lost ca. 6- to 20-fold activity in regard to the corresponding L-alanine analogues.

5.5.2 Enzymatic studies using Carboxypeptidase Y on 6OMe2'CMcG ProTides.

Described in the following paragraph are the enzymatic assays performed on the 6OMe2'CMcG phosphoramidate derivatives reported in the previous section of this Chapter, using procedures reported in Chapter 4.

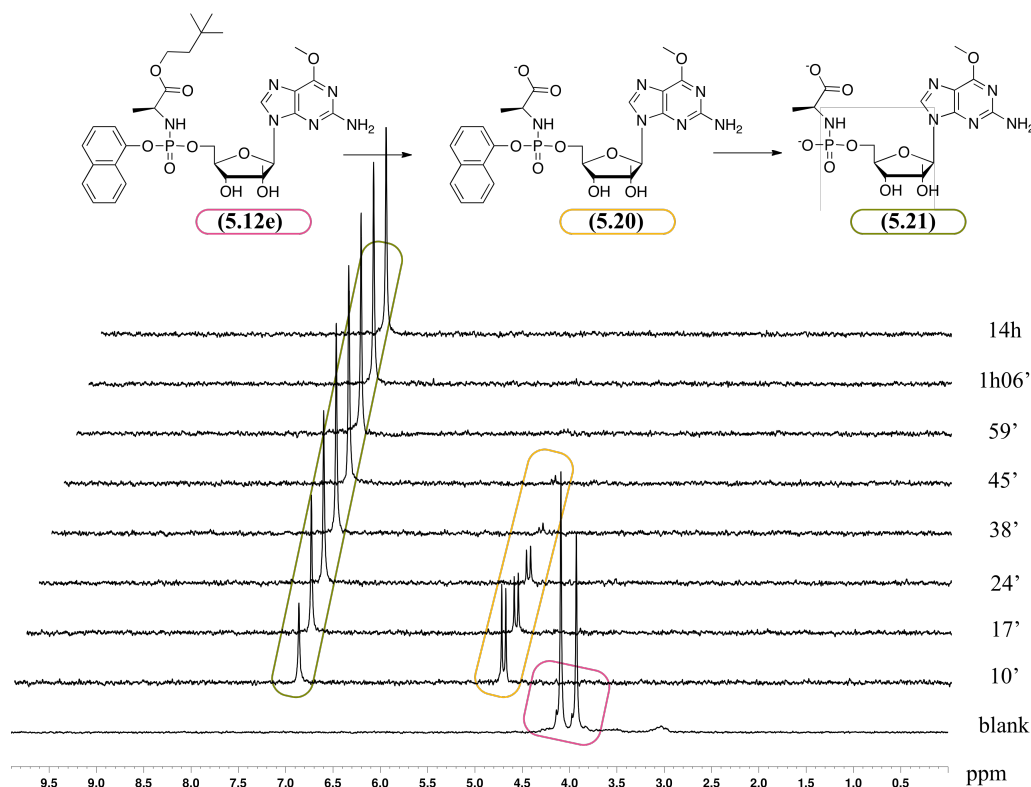


Figure 5.10. Carboxypeptidase Y mediated hydrolysis of **5.12e**, followed by ^{31}P NMR.

A first experiment was performed with the α -naphthyl L-alanine 3,3-dimethyl-1-butyl ester 5'-phosphoramidate **5.12e** (Figure 5.10). In the blank ^{31}P NMR two diastereoisomer (ca. 1:1 ratio) signals were observed ($\delta_{\text{P}} = 3.92$ and 4.10 ppm). Once the enzyme was added, the compound was quickly hydrolysed to the intermediate (**5.20**, $\delta_{\text{P}} = 4.53$ and 4.85 ppm) lacking the ester moiety, which appeared in 1:1 ratio. It can be noticed that processing of both diastereoisomers proceeded at the similar rate. At the 10-minute time point, a peak corresponding to the final aminoacyl intermediate (**5.21**, $\delta_{\text{P}} = 6.94$ ppm) was formed (Figure 5.10). The half-life of the compound **5.12e** is estimated to be less than 5 min.

Carboxypeptidase assay performed on L-valine derivative **5.12o** shows good conversion of the parent ProTide (Figure 5.11).

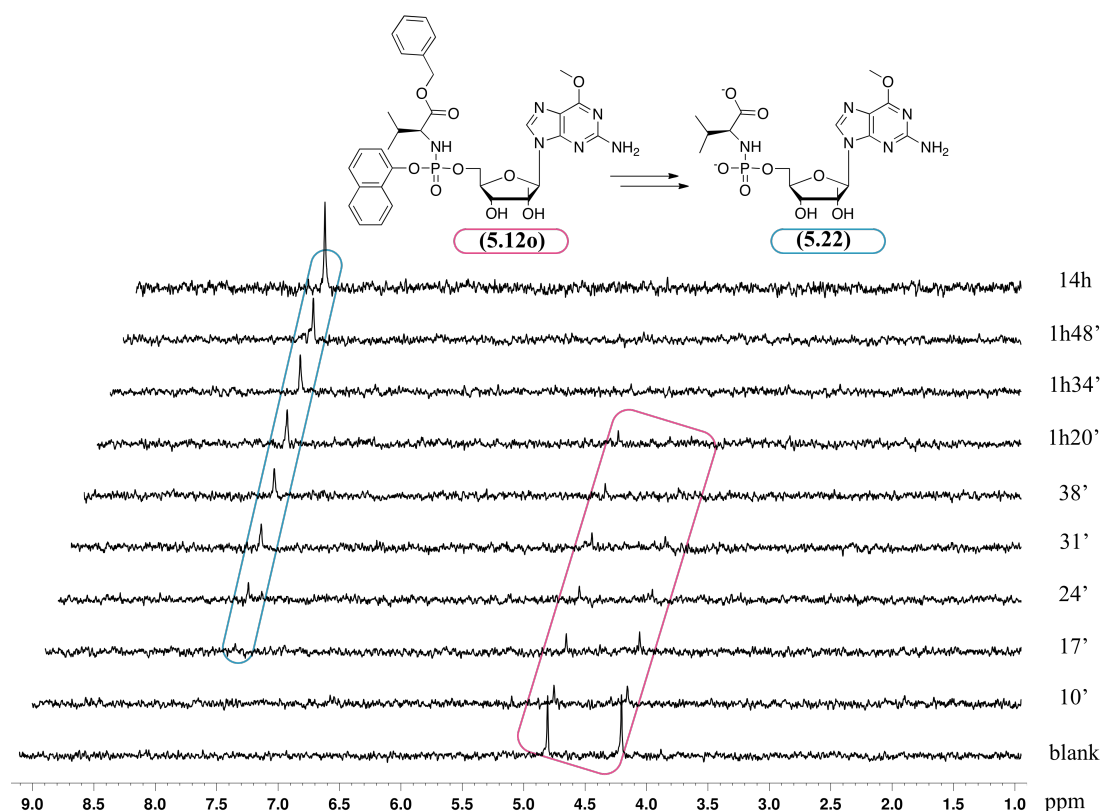


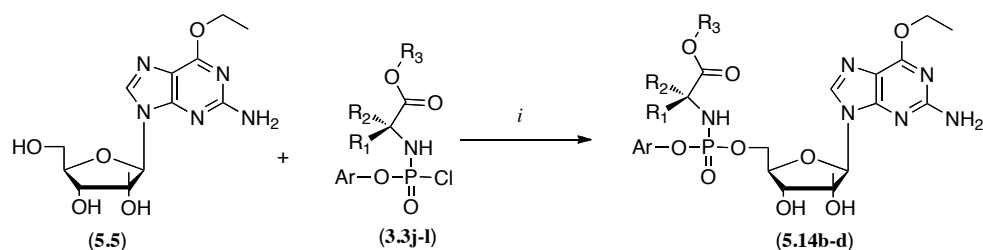
Figure 5.11. Carboxypeptidase Y mediated hydrolysis of **5.12o**, followed by ^{31}P NMR.

After addition of carboxypeptidase Y, the formation of new peak ($\delta_{\text{P}} = 7.38$ ppm) corresponding to the chemical structure of **5.22** was observed within 17 min. Completion of the processing to the aminoacyl intermediated **5.22** was achieved after ca. 1.5 h.

The biological results reported for these two compounds supported by the enzymatic data can be taken as an early indication that the 2-amino-6-methoxy-9-(2'-C-methyl- β -D-ribofuranosyl)purine ProTides successfully improved the intracellular delivery of the eventual 5'-triphosphate pharmacophore of 2'-C- β -methylguanosine.

5.5.3 Synthesis and biological evaluation of 6-O-ethyl-2'-C- β -methylguanosine phosphoramidates.

Further exploration of the 2-amino-6-O-ethyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine ProTides lead to the small series of L-alanine derivatives. Compounds were prepared via coupling reaction with different phosphorochloridates **3.3j-l**, in dry THF in the presence of *t*BuMgCl (Scheme 5.5). All phosphoramidates were isolated as roughly equimolar mixtures of diastereoisomers at the phosphate, in moderate to good yields (13-22%).



Reagents and Conditions: i) appropriate phosphorochloridate, *t*BuMgCl, dry THF, rt, overnight.

Scheme 5.5. General method for the synthesis of aryloxyphosphoramidates of 6-O-ethyl-2'-C-methylguanosine using *t*BuMgCl.

6-O-Ethyl-2'-C- β -methylguanosine ProTides were evaluated by Inhibitex Inc. against hepatitis C virus *in vitro* using a replicon assay.

Again, the phosphoramidate technology allowed us to convert a poorly active nucleoside into ProTides with nM activity (Table 5.6). All newly synthesised phosphoramidates were 50- to ~900-fold more potent than the parent nucleoside. The 900-fold boost in activity of L-alanine-2,2-dimethylpropyl derivative **5.14a**, is the highest one observed so far among all synthesised phosphoramidates of 2'CMcG and closely related nucleoside analogues. L-Alanine benzyl ester analogue **5.14b** was approximately 4-times less potent than the most active **5.14a**, nevertheless this compound was circa 200-fold more efficient as an inhibitor of viral replication than the parent nucleoside analogue **5.5** (Table 5.6, Figure 5.12a).

| Cpd | Ar | R1 | R2 | AA | R3 | ClogP | EC ₅₀ (μM) | CC ₅₀ (μM) |
|--------------|--------|-----------------|----|-------|--------------------|-------|-----------------------|-----------------------|
| 5.5 | - | - | - | - | - | -0.1 | 8.8 | >100 |
| 5.14a | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3.8 | 0.01 | 10 |
| 5.14b | α-Naph | CH ₃ | H | L-Ala | tetrahydropyranyl | 1.6 | 0.08 | 13 |
| 5.14c | α-Naph | CH ₃ | H | L-Ala | benzyl | 3.7 | 0.04 | 12 |
| 5.14d | α-Naph | CH ₃ | H | L-Ala | S-phenylethyl | 4.0 | 0.09 | 12 |

AA: Amino acid; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 5.6. Summary of the anti-HCV activity 6OEt2'CMeG ProTides and their calculated ClogP values.

The two phosphoramidates bearing tetrahydropyranyl- **5.14b** and *S*-phenylethyl esters **5.14d** were the least active across this small family of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl-β-D-ribofuranosyl)purine ProTides, showing circa 8-fold decrease in activity comparing to **5.14a** (Figure 5.12a).

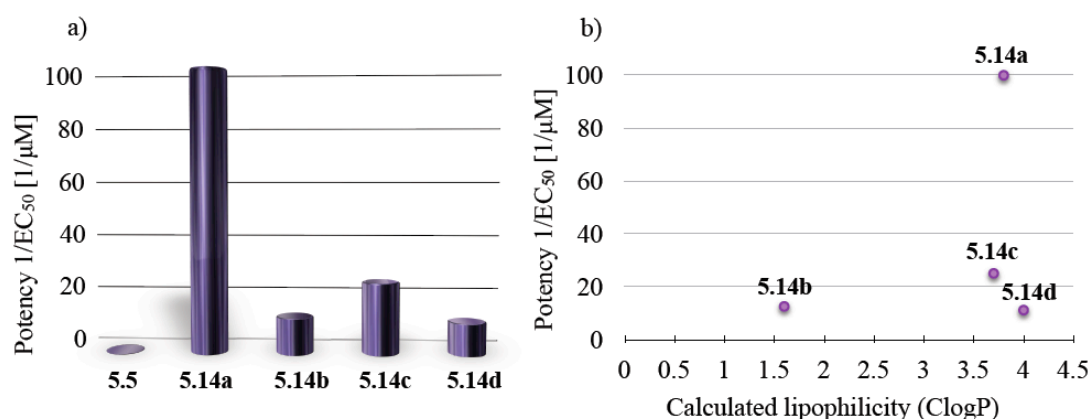


Figure 5.12. a) Potency (1/EC₅₀) data plot of L-alanine ProTides of 6OEt2'CMeG; b) Potency (1/EC₅₀) vs. calculated lipophilicity data plot of L-alanine ProTides of 6OEt2'CMeG

Similarly like in the case of 6OMe2'CMeG ProTides, compounds with calculated lipophilicity values in a range between 2 and 4, exhibited the best antiviral activity (Figure 5.12b). Of course it has to be remembered that logP is only one of the factors that contributes to a successful compound. There are other very important features including: the ease of enzymatic activation and ability to release corresponding 5'-monophosphate, which can be subsequently processed to the active 5'-triphosphate species.

5.5.4 Enzymatic and cell lysate studies on 6OEt2'CMeG ProTides.

To confirm the possible mode of action of these 6OEt2'CMeG phosphoramidates, enzymatic assays were performed in order to investigate the activation pathway (Figure 5.13 and Figure 5.14).

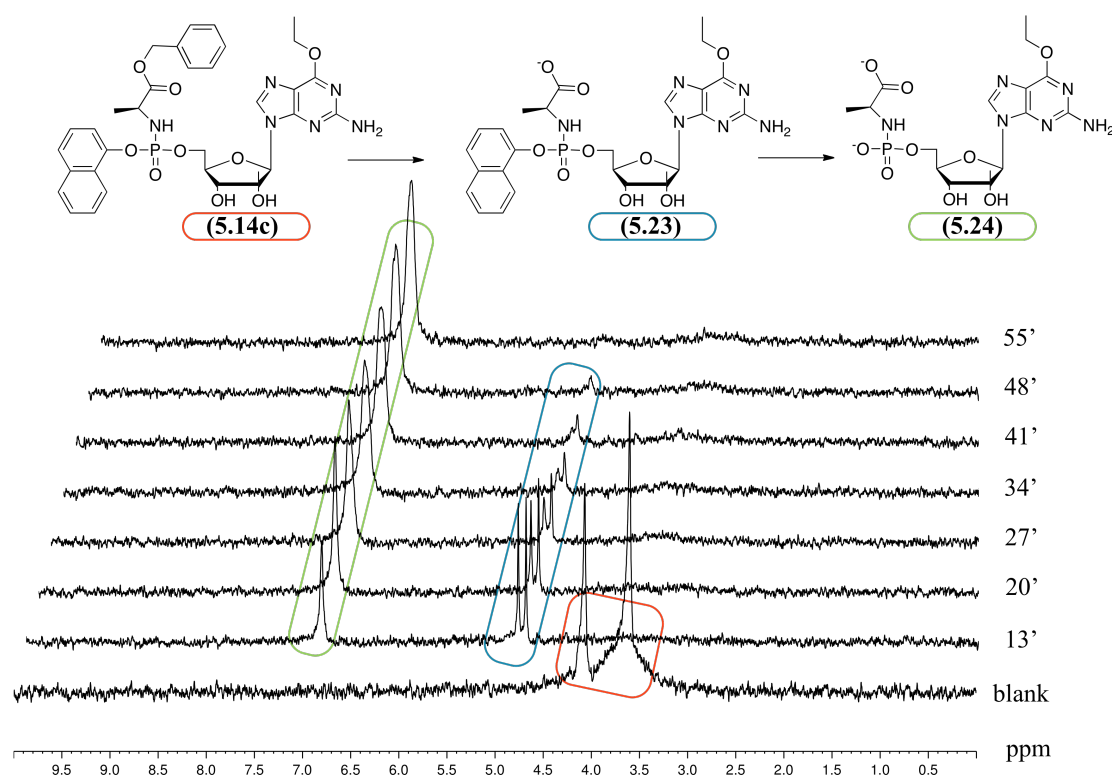
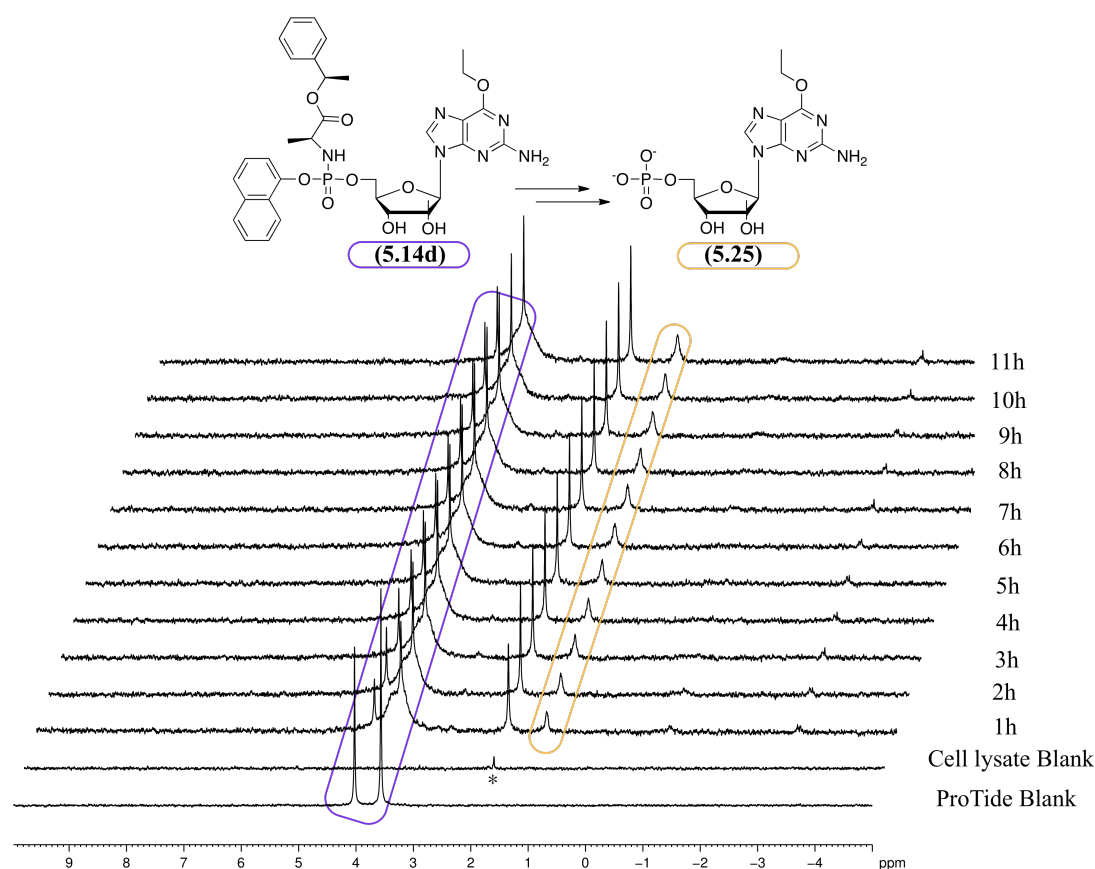


Figure 5.13. Carboxypeptidase Y mediated hydrolysis of **5.14c**, followed by ³¹P NMR.

In the first instance the carboxypeptidase Y assay was performed on naphthyl benzylalanine analogue **5.14c** (Figure 5.13). During the first 13 min after enzyme addition, the starting material **5.14c** ($\delta_P = 3.62$ and 4.11 ppm) was quickly hydrolysed to the intermediate lacking ester moiety **5.23** ($\delta_P = 4.69$ and 4.83 ppm). Notably both diastereoisomers were processed with roughly similar efficacy. The estimated half-life of **5.14c** was less than 5 min. The final product of the hydrolysis, achiral aminoacyl phosphate **5.24** is represented by a single peak at $\delta_P = 6.95$ ppm. Efficient activation of **5.14c** is consistent with the replicon data reported for this compound, indicating that carboxypeptidase assay is a useful tool for the *in vitro* probing of the first steps of ProTides activation.

The release of aminoacyl intermediate, the final product of carboxypeptidase Y mediate hydrolysis is considered as a first stage of ProTides activation. In order to gain its antiviral activity this particular intermediate needs to be hydrolysed to the 5'-monophosphate, which subsequently could undergo kinase-dependent activation to the corresponding 5'-triphosphate.

The ability of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine ProTides to release 5'-monophosphate was investigated in Huh7 cell lysate and the corresponding data are reported in Figure 5.14.



* Phosphate peak present in the cell lysate, remains unchanged during the experiment.

Figure 5.14. Formation of 5'-monophosphate from **5.14d** in cell lysate assay, followed by ^{31}P NMR.

Compound **5.14d** was incubated in the presence of the Huh7 cell lysate (10^7 cells) in acetone- d_6 and Trizma buffer (pH 7.6) at 37 °C. ^{31}P NMR spectra were recorded in 1 h intervals for 11 h. During the first hour of incubation, the appearance of new peak at $\delta_{\text{P}} = 0.98$ ppm was observed. The presence of that species can indicate successful liberation of **5.25** from the parent ProTide. In fact, this species has exactly the same chemical shift as chemically synthesised 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-

ribofuranosyl) purine 5'-monophosphate under the same experimental conditions. During the hydrolysis the presence of aminoacyl intermediate **5.24** was not observed, suggesting that this metabolite is rapidly deaminated resulting in the formation of **5.25**.

5.5.5 ProTides anti-HCV activity studies in the presence of adenosine and adenylate deaminase inhibitors.

As already discussed in the previous sections of this Chapter, the C6-modified nucleosides like **5.4** or **5.5** require deaminase mediated cleavage to obtain their full antiviral potency. To further investigate these findings specific antiviral assays were performed. The anti-HCV potency values of selected ProTides were determined in genotype 1b replicon cells, in the presence or absence of 2 μM EHNA or 40 μM pentostatin (deoxycofymycin, dCF) and evaluated by Inhibitex Inc. The corresponding data are reported in Figure 5.15.

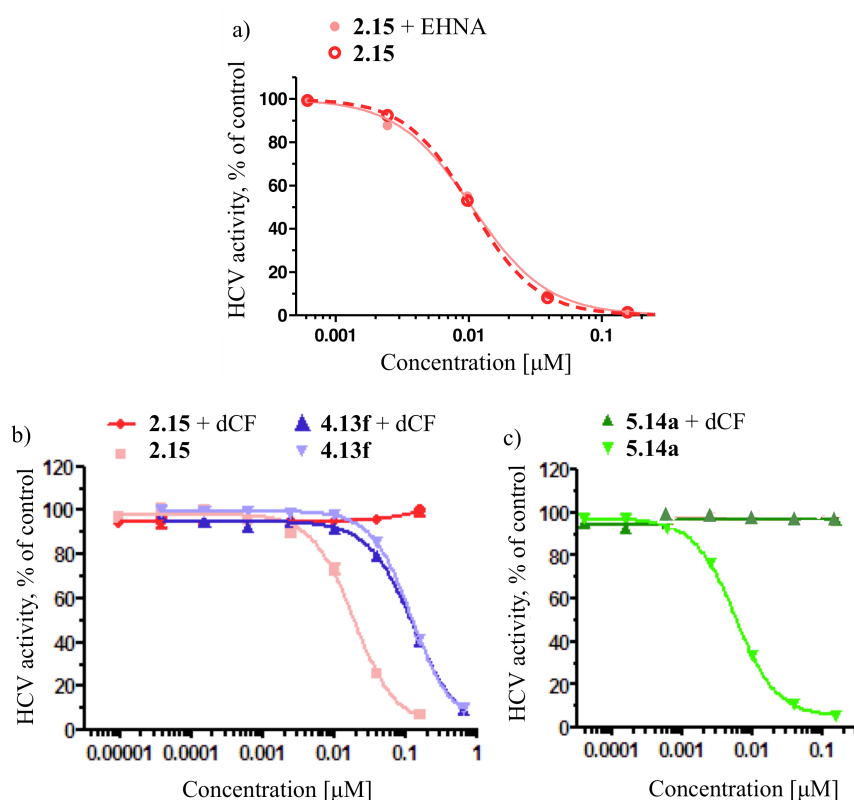
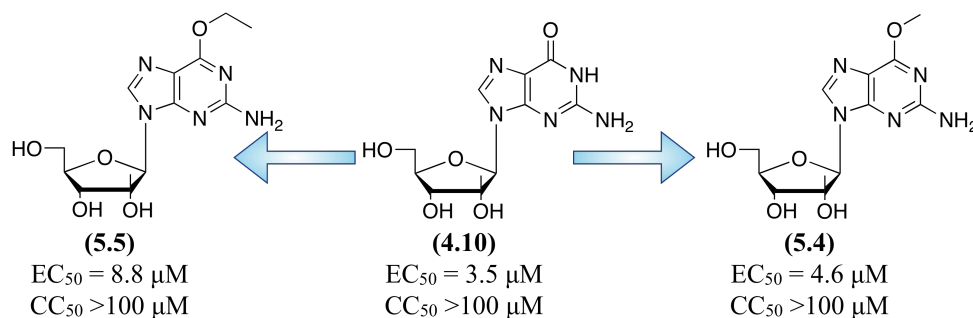


Figure 5.15. Effect of EHNA and dCF on activity of **4.13f**, **2.15** and **5.14a** ProTides.

In the first instance effect of EHNA on activity of naphthyl-L-alanine-2,2-dimethylpropyl 6-*O*-methyl-2'-*C*-methylguanosine **2.15** was investigated. As can be seen in Figure 5.15a, EHNA has no effect on antiviral potency of **2.15**. This data, with adenosine deaminase specific inhibitor suggests that the primary route of activation of **2.15** involves direct conversion of the phosphoramidate to the corresponding 5'-monophosphate then di- and triphosphate with negligible contribution of the nucleoside metabolite. Influence of pentostatin, a known inhibitor of ADA, AMPDA and ADAL1, on antiviral activity of **4.13f**, **2.15** and **5.14a** is shown in Figure 5.15b and c. Each of the ProTides bearing modifications in the C6 position (**2.15** and **5.14a**), loses entirely their anti-HCV activity in the presence of pentostatin, while **4.13f**- the 2'-*C*-methylguanosine analogue retain its potency. These findings clearly confirm an absolute need for the hydrolysis of C-6 substituent in order to be active, and that this happens at the nucleotide level.

5.5.6 Effect of 6-*O*-alkylation on activity of 2'-*C*- β -methylguanosine phosphoramidates.

In vitro replicon based assay showed that both parent nucleosides 2-amino-6-methoxy-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine and 2-amino-6-ethoxy-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine displayed similar activity as 2'-*C*- β -methylguanosine (Scheme 5.6). With **5.5** being ca. 2-fold less active than **4.10**.



Scheme 5.6. Comparison of EC_{50} of 6OEt2'CMeG, 2'CMeG and 6OMe2'CMeG.

The most probable explanation for that difference is fact that both **5.4** and **5.5** require additional hydrolytic step before being converted to the corresponding 2'-*C*- β -methylguanosine 5'-triphosphate. Lower EC_{50} value exhibited by the 6-*O*-ethyl is in

accordance with adenosine/adenylate deaminase substrate specificity, which was discussed in the section 5.2.3 of this Chapter.

All three nucleoside analogues were found to be non-toxic in the cell based assay.

Comparing compounds on the ProTide level it can be noticed that the introduction of the 6-methoxy moiety provided 2-5-fold improvement in the potency comparing to the corresponding 2'CMeG ProTides (Table 5.7). The analogous 6-ethoxy phosphoramidates were found to be active against HCV replicon with sub-micromolar activity, comparable to the 6-methoxy analogues (Table 5.7).

| Nucleoside | L-Alanine | | | | | |
|------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | 2,2-dimethylpropyl | | benzyl | | S-phenylethyl | |
| | EC ₅₀ [μM] | CC ₅₀ [μM] | EC ₅₀ [μM] | CC ₅₀ [μM] | EC ₅₀ [μM] | CC ₅₀ [μM] |
| 2'CMeG | 0.05 | >100 | 0.06 | >100 | 0.08 | >100 |
| 6OMe2'CMeG | 0.01 | 7 | 0.03 | 12 | 0.03 | 14 |
| 6Et2'CMeG | 0.01 | 10 | 0.04 | 12 | 0.09 | 12 |

Table 5.7. Comparison between anti-HCV activity of analogous ProTides bearing different C-6 substitutions.

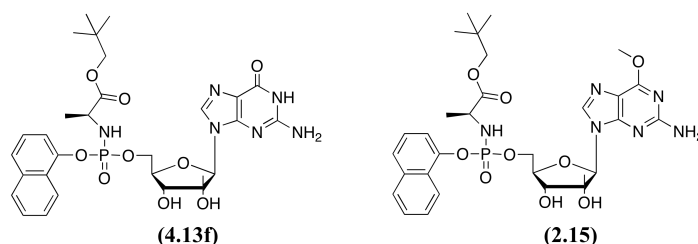
These results confirm the 6-*O*-alkylation principle of concept. It is suggested that the improved lipophilicity of the base unit and application of the ProTide technology, can increase both: cellular uptake and metabolic conversion to the active 2'CMeG 5'-triphosphate, resulting in enhanced anti-HCV inhibitory activity.

5.5.7 Effect of 6-*O*-alkylation on membrane permeation.

Effect of increased lipophilicity on cellular uptake was investigated in Caco-2 studies. The most potent phosphoramidate in the 2-amino-6-methoxy-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine family **2.15** was evaluated alongside with 2'-*C*-β-methylguanosine analogue **4.13f** (Table 5.8).

Caco-2 permeation studies were performed by Dr M. Gumbleton and Dr M. Smith at Cardiff University.

Permeation was measured as % transport in 80 minutes across Caco-2 cells grown for 27 days (resistance 600 ohms/cm²), measured apical to basal.



| Cpd | Replicon 1b | | | ClogP | Caco-2 % |
|--------------|-----------------------|-----------------------|-----------------------|-------|----------|
| | EC ₅₀ [μM] | EC ₉₀ [μM] | CC ₅₀ [μM] | | |
| 4.13f | 0.05 | 0.27 | >100 | 1.3 | <0.5 |
| 2.15 | 0.01 | 0.038 | 7 | 3.1 | 2.7 |

Table 5.8. Anti-HCV activity, cytotoxicity, calculated lipophilicity and observed Caco-2 permeation for **4.13f** and **2.15**.

From the Caco-2 studies it is clear, that introduction of 6-*O*-methyl modification increased calculated lipophilicity by ca. 6-fold (Table 5.8) comparing to the corresponding 2'-*C*-β-methylguanosine derivative **4.13f**. This translates into ≥5-fold improvement in membrane transport as measured by Caco-2 permeation. Perhaps as a consequence 5-fold improvement in antiviral activity against HCV replicon 1b for **2.15** was observed.

These data further support the notion that increase of lipophilicity of nucleoside analogues and implementation of ProTide technology can enhance cellular uptake and result in improved potency.

5.6 Intracellular levels of 2'-*C*-methylguanosine triphosphate – *in vitro* studies.

5.6.1 Intracellular levels of 2'-*C*-β-methylguanosine triphosphate in replicon 1b cells – *in vitro* studies.

In order to determine whether 6OMe2'*C*MeG ProTides can be converted intracellularly to the 2'-*C*-methylguanosine TP, replicon 1b cells were incubated in the presence of **2.15** at concentrations required to produce 50%, 90% and 2x 90% inhibition of viral replication (EC₅₀ = 0.01 μM, EC₉₀ = 0.04 μM and 2x EC₉₀ = 0.08 μM, respectively). After 6 h of exposure to **2.15**, cells were harvested and intracellular levels of 2'-*C*-methylguanosine triphosphate were measured (Table 5.9).

| Concentration of 2.15 [nM] | Intracellular 2'CMeGTP [pmol/1 x 10 ⁶ cells] | Estimated equivalent in the liver [pmol/g tissue] |
|--------------------------------------|--|--|
| 10 (1x EC ₅₀) | 0.84 ± 0.36 | 84 |
| 40 (1x EC ₉₀) | 2.43 ± 0.42 | 243 |
| 80 (2x EC ₉₀) | 4.97 ± 0.80 | 497 |

All values are means ± standard deviation from six determinations.

Table 5.9. Intracellular levels of 2'CMeGTP in replicon cells resulted from the incubation with **2.15**.

At 0.01 µM **2.15**, the concentration of 2'CMeGTP produced in replicon cells was 0.84 ± 0.36 pmol/1 x 10⁶ cells, what considering the liver cellularity would correspond to the 84 pmol/g of liver tissue. Incubation of replicon cells with 0.04 µM concentration of **2.15** (1x EC₉₀), resulted in formation of 2.43 ± 0.42 pmol/1 x 10⁶ cells of intracellular 2'CMeGTP.

This data would correspond to the 243 pmol/g tissue of 2'-C-methylguanosine 5'-triphosphate. This amount would be expected to eradicate 90% of viral load in the liver tissue.

5.6.2 Intracellular levels of 2'-C-β-methylguanosine triphosphate in human hepatocytes – *in vitro* studies.

Further investigation of triphosphate production was carried out in primary human hepatocytes. Studies were performed and evaluated by Inhibitex Inc.

ProTides were incubated in primary human hepatocytes at an arbitrary concentration of 2 µM. Triphosphate levels were measured over a period of 24 h at 2, 6, 12 and 24 h time points. Additionally levels of 2'CMeGTP resulting from the incubation of 2'-C-methylguanosine **4.10** and 6-*O*-methyl-2'-C-methylguanosine **5.4** during 12 h and 24 h were also measured.

Several structurally different ProTides were chosen for the study, including L-alanine (**2.15**, **5.12c**, **5.12h**) and L-valine (**5.12m**) derivatives and the corresponding results are shown in Figure 5.16.

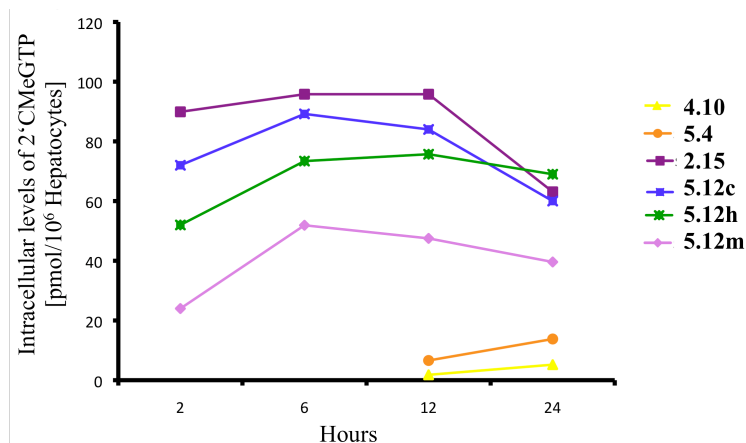
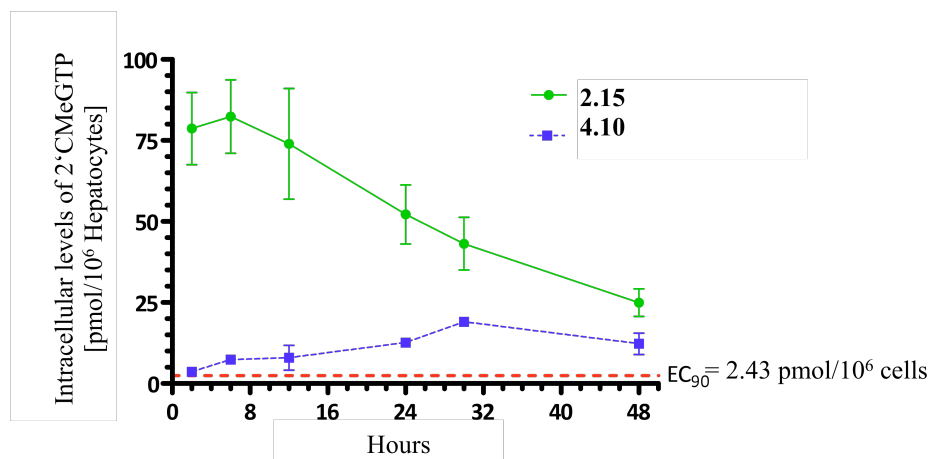


Figure 5.16. 24h time course of 2'CMeGTP production in primary human hepatocytes of selected ProTides **5.12a**, **5.12d**, **5.12i**, **5.12n** and parent nucleosides **4.10** and **5.4**.

Incubation of all phosphoramidates resulted in formation of high amounts of triphosphate within 6 h of incubation. In comparison incubation of both nucleoside analogues **4.10** and **5.4** resulted in rather poor triphosphate levels. Looking at L-alanine and L-valine analogues, it can be clearly seen that the latter produce significantly less 2'CMeG triphosphate. These data correlate with the ease of activation observed for the appropriate L-alanine and L-valine phosphoramidates and their EC_{50} values. The highest levels of 2'-C- β -methylguanosine 5'-triphosphate were obtained from the incubation of **2.15**, followed by **5.12c** and **5.12h**.

Compound **2.15** was chosen for prolonged (48 h) incubation studies (Figure 5.17). The data show that L-alanine-2,2-dimethylpropyl phosphoramidate can produce a substantial amount of 2'-C-methylguanosine triphosphate comparing with the nucleoside analogue **4.10**. The maximum concentration (C_{max}) of TP formed during the incubation of **2.15** was ca. 83 pmol/ 10^6 cells (twice as much as obtained from the corresponding 2'CMeG ProTide **4.13f**) and has formed within first 8 h, and then I slowly decays.

On the other hand 2'CMeG was found to slowly build up triphosphate levels, with significantly lower and delayed C_{max} of around 20 pmol/ 10^6 cells at 30 h (Figure 5.17).



EC₉₀: Defined as the amount of intra cellular triphosphate necessary to achieve 90% inhibition in the HCV replicon assay.

Figure 5.17. Levels of 2'CMeGTP produced from ProTide **2.15** and nucleoside **4.10** in primary human hepatocytes system over 48h incubation period.

Levels of 5'-triphosphate resulted from the incubation of **2.15** in primary human hepatocytes were more than 30-times higher than the concentration required to eradicate 90% of the viral load, indicating undeniable potential of **2.15** as an anti-HCV agents.

Based on the exceptional anti-HCV activity of **2.15**, improved permeability and ability to produce substantial levels of 2'-C-methylguanosine-5'-triphosphate, the compound was chosen for the further *in vitro* and *in vivo* studies supporting its selection and subsequent progression as a clinical candidate. Data regarding biological evaluation of **2.15** and current status of the compound are reported in Chapter 6.

5.7 Conclusions.

In conclusion, a series of novel C6-modified 2'-C-methylguanosine analogues has been synthesised. The rational behind the design was to introduce modification that could be later on hydrolysed and therefore act as a pro-moiety. It was found that at the nucleoside level, adenosine deaminase was able to 'deaminate' most of the synthesised nucleosides. ADA inhibition studies confirmed absolute need for these compounds to be hydrolysed in order to act as inhibitors of viral replication. All synthesised nucleoside analogues exhibited improved calculated lipophilicity, however their antiviral activities were lower than that corresponding to the 2'-C-

methylguanosine. To enhance antiviral activity, ProTide technology was applied to all synthesised nucleoside analogues. In almost every case, the newly synthesised phosphoramidates were more active than the parent nucleoside, often by >30-fold and in many cases by 300 to 800-fold. Structure activity relationship was extensively studied, including modification in ester, aryl and amino acid moiety. A combination of different esters and amino acids resulted in very potent (nM) compounds, suitable for further preclinical development. Comparative Caco-2 studies between C6-modified ProTide and their corresponding 2'-C-methylguanosine analogue, revealed that the C6-modified compound was able to cross cell membrane more efficiently (enhanced cellular uptake) and as a consequence improved anti-HCV activity was seen. Ability of the synthesised ProTides to produce substantial levels of 2'-C-methylguanosine triphosphate *in vitro* confirmed their undisputed potential. All collected data suggest that ProTides of 6-O-methyl-2'-C-methylguanosine represent a very interesting class of compounds for the treatment of HCV infections, with α -naphthyl-L-alanine-2,2-dimethylpropyl ProTide showing the biggest promise. This particular compound was further progressed into clinical development as **INX-189**.

References:

1. Lambe CU, Averett DR, Paff MT, Reardon JE, Wilson JG, Krenitsky TA. 2-Amino-6-methoxy Arabinoside: An agent for T-cell malignancies. *Cancer Res* **1995**, 55, 3352-3356.
2. WO2004/052899 A2, PCT/US2003/39643
3. Eldrup AB, Allerson CR, Bennett CF, Bera S, Bhat B, Bhat N, Bosserman MR, Brooks J, Burlein C, Carroll SS, Cook DP, Getty KL, MacCross M, McMasters DR, Olsen DB, Prakash TP, Prhave M, Song Q, Tomassini JE, Xia J. Structure-activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J Med Chem* **2004**, 47, 2283-2295.
4. Ding Y, Girardet JL, Hong Z, Hai VCH, An H, Koh Y, Shaw SZ, Zhong W. Synthesis of 9-(2-b-C-methyl-b-D-ribofuranosyl)-6-Substituted purine derivatives as inhibitors of VCH RNA replication. *Bioorg Med Chem Lett* **2005**, 15, 709-713.
5. Gunic E, Chow S, Rong F, Ramasamy K, Raney A, Li DY, Huang J, Hamatake RK, Hong Z, Girardet JL. 6-Hydrazinopurine 2'-Methyl ribonucleosides and their 5'-monophosphate prodrugs as potent hepatitis C virus inhibitors. *Bioorg Med Chem Lett* **2007**, 17, 2456-2458.
6. Hanna NB, Bhattacharya BK, Robins RK, Avery TL, Revankar GR. Sulfinosine congeners: synthesis and antitumor activity in mice of certain N9-alkylpurines and purine ribonucleosides. *J Med Chem* **1994**, 37, 177-183.
7. Ikejiri M, Oshima T, Kato K, Toyama M, Murata T, Shimatohno K, Maruyama T. 5'-O-Masked 2'-deoxyadenosine analogues as lead compounds for hepatitis C virus (HCV) therapeutic agents. *Bioorg Med Chem* **2007**, 15, 6882-6892.
8. Harada T, Oku A. Reaction of alkoxides with phenylthiocarbene: a novel oxy-anionic substituent effect on the carbon-hydrogen insertion of carbenes. *J Am Chem Soc* **1981**, 103, 5965-5967.
9. WO2004/024082, PCT/US2003/028594
10. Ikehara M, Fukui T. Studies of nucleosides and nucleotides. LVIII. Deamination of adenosine analogs with calf intestine adenosine deaminase. *Biochim Biochem Acta* **1974**, 338, 512-519.

11. Santaniello E, Ciuffreda P, Alessandrini L. Synthesis of modified purine nucleosides and related compounds mediated by adenosine deaminase (ADA) and adenylyate deaminase (AMPDA). *Synthesis* **2005**, 4, 509-526.
12. Murakami E, Bao H, Mosley RT, Du J, Sofia MJ, Furman PA. Adenosine deaminase like protein 1 (ADAL1): Characterization and substrate specificity in the hydrolysis of N6- or O6-substituted purine or 2-aminopurine nucleoside monophosphates. *J Med Chem* **2011**, 54, 5902-5914.
13. Farrow SN, Jones AS, Kumar A, Walker RT, Balzarini J, De Clercq E. Synthesis and biological properties of novel phosphotriesters: a new approach to the introduction of biologically active nucleotides into cells. *J Med Chem* **1990**, 33, 1400-1406.

CHAPTER SIX

Identification of INX-189: from bench to the clinical trials.

6.1 Characterisation of INX-08189.

During our ongoing effort to identify new, potent inhibitors of HCV replication, we discovered α -naphthyl-L-alanine-2,2-dimethylpropyl 6-*O*-methyl-2'-*C*- β -methylguanosine phosphoramidate **2.15**. This compound exhibited extraordinary anti-HCV activity along with other desirable properties such as cell permeability and the ability to produce high levels of 2'-*C*- β -methylguanosine 5'-triphosphate in primary human hepatocytes. Based on all available data, it was decided that compound **2.15** – **INX-08189 (INX-189, BMS-094)**, would be advanced in to further preclinical development, supporting its selection as a clinical candidate. Much of the work was done by our collaborators, as acknowledged below.

6.2 Biological evaluation and carboxypeptidase Y studies of separated isomers of INX-189.

Most of the phosphoramidate ProTides are represented by a mixture of diastereoisomers at the phosphorus centre. PSI-7977 as already described in Chapter 2, is a chirally pure (*Sp*) isomer of β -D-2'-deoxy-2'-fluoro-2'-*C*-methyluridine phosphoramidate. The reason why only one of the isomers was advanced into clinical trials was significant difference in potency between two separated isomers ~100-fold (Table 6.1).¹

| Cpd | Isomer | Clone A replicon | | ET replicon | |
|---------|---------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| | | EC ₉₀ [μ M] | CC ₉₀ [μ M] | EC ₉₀ [μ M] | CC ₉₀ [μ M] |
| PSI7851 | mixture | 0.48 | >100 | 0.22 | >100 |
| PSI7976 | Rp | 2.96 | >100 | 1.85 | >100 |
| PSI7977 | Sp | 0.28 | >100 | 0.17 | >100 |

EC₉₀: 90% effective concentration or compound concentration required to inhibit HCV replication by 90%;
CC₉₀: 90% cytotoxic concentration.

Table 6.1. Anti-HCV activity and cytotoxicity of PSI7851, PSI7977 and PSI7976 phosphoramidates.¹

Given the considerable interest in **INX-189** it was decided to separate the two diastereoisomers. Isomer separation was performed by Chiral Technologies Inc. The absolute configuration of each of the two diastereoisomers was determined by vibrational circular dichroism (VCD) by BioTools Inc., and later on confirmed by the crystal structure.

Pure isomers were tested against HCV in replicon assay by Inhibitex Inc. Interestingly the two separated isomers shown only 2-fold difference in antiviral potency (Table 6.2).

| Cpd | Replicon 1b | δ ^{31}P [ppm] | $T_{1/2}$ [min] |
|-------------------|------------------------------------|--------------------------------|-----------------|
| | EC_{50} [μM] | | |
| INX-189 Sp | 0.019 | 4.23 | 17 |
| INX-189 Rp | 0.044 | 4.28 | 20 |

Table 6.2. Data on separated isomers of **5.12a- INX189**.

It was hypothesised that this comparable potency may arise from the similar rate of activation of both isomers. To investigate the initial cleavage of amino acid ester and subsequent liberation of aminoacyl intermediate, carboxypeptidase Y assay was performed (Figure 6.1 and 6.2) and related data are presented in Table 6.2.

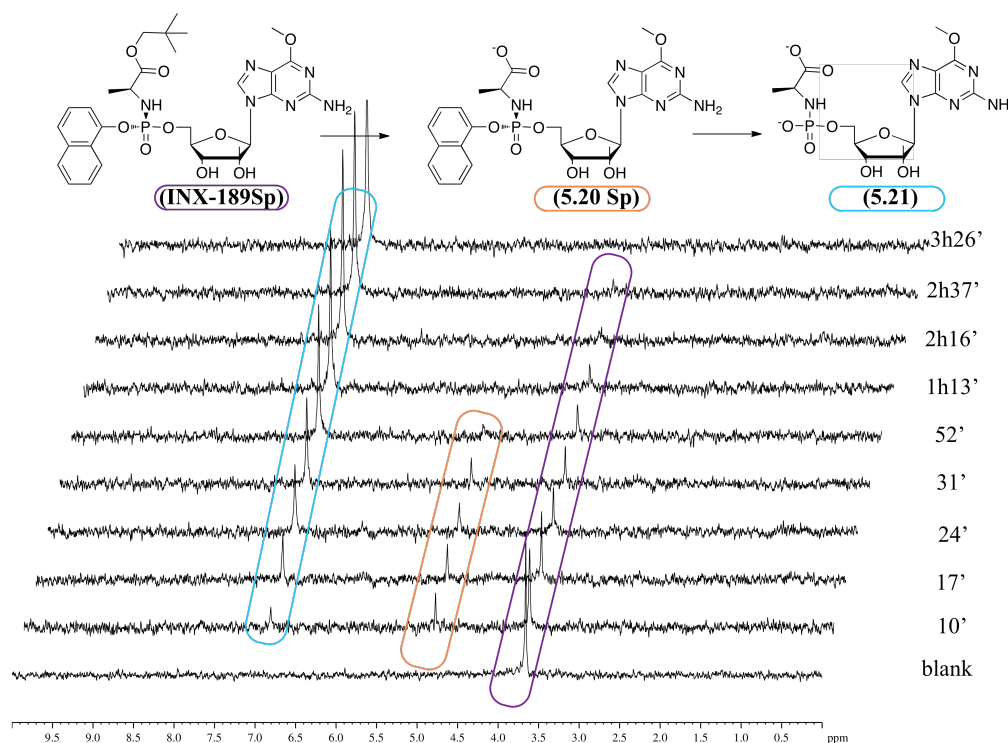


Figure 6.1. Carboxypeptidase Y mediated hydrolysis of **INX-189 Sp**, followed by ^{31}P NMR.

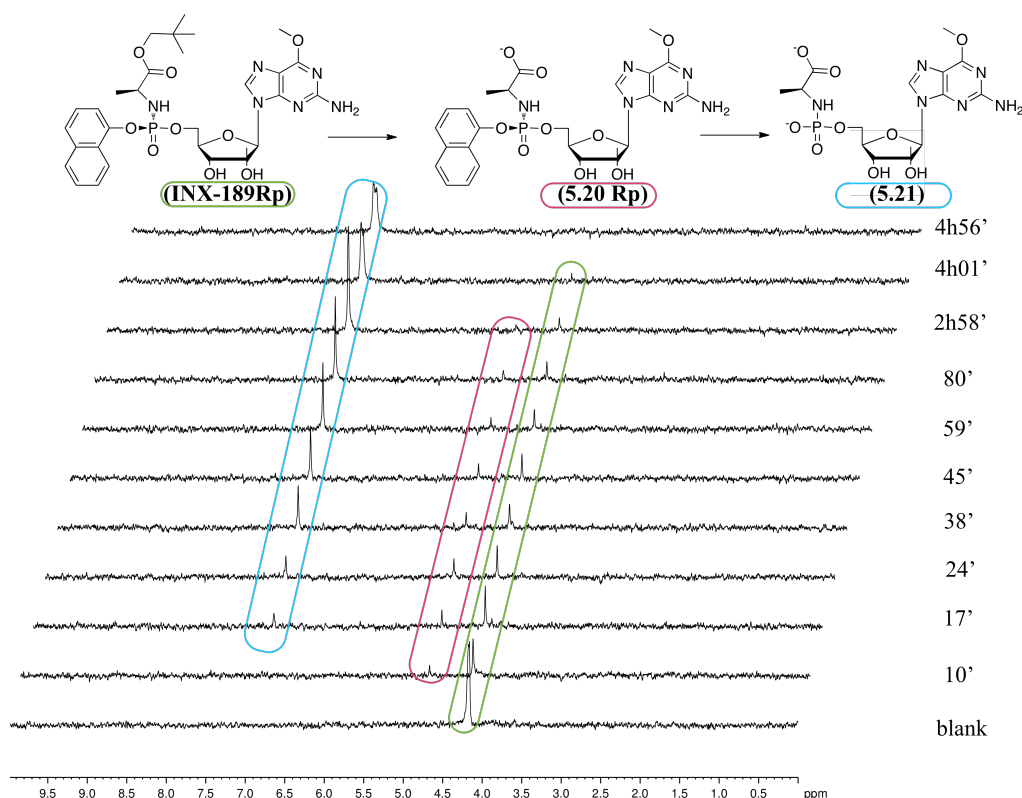


Figure 6.2. Carboxypeptidase Y mediated hydrolysis of **INX-189 Rp**, followed by ^{31}P NMR.

Data reported in the Figure 6.1 and 6.2 represent ^{31}P MNR spectra recorded every 7 minutes over 5 hours incubation period with carboxypeptidase Y in acetone- d_4 and Trizma buffer (pH 7.6). In the case of **INX-189 Sp** isomer (Figure 6.1), after addition of the enzyme, the parent phosphoramidate ($\delta_{\text{P}} = 3.76$ ppm) is cleaved to the free amino acid carboxylate ($\delta_{\text{P}} = 4.92$ ppm). The final product of the activation is represented by the single peak at 6.95ppm and corresponds to the aminoacyl phosphate. The aminoacyl intermediate started to form within first 10 min and full conversion of the parent ProTide was achieved within 3 h. The half-life for the separated Sp isomer was estimated as ca. 17 min. Looking at carboxypetidase Y assay of **INX-189 Rp** isomer ($\delta_{\text{P}} = 4.27$ ppm), it can be clearly noticed that the initial hydrolysis of the ester moiety proceeds at a slightly slower rate comparing to the **INX-189 Sp** analogue. Interestingly, the free amino acid carboxylate intermediated ($\delta_{\text{P}} = 4.82$ ppm) which has formed, has ^{31}P shift more upfield than the corresponding Sp analogue. Final aminoacyl species ($\delta_{\text{P}} = 6.95$ ppm), started to form within the first 17 min and full conversion of the **INX-189 Rp** derivative was accomplished within ca. 4.5 h. The estimated half-life of the Rp

isomer was 20 min. These findings are consistent with the replicon data, showing that the Sp isomer, which is processed somewhat faster, is also found to be 2-fold more active.

Taking in consideration all available data, it was decided not to separate **INX-189** to the single isomers, and use it for the further evaluation as a diastereomeric mixture.

6.3 Activity of INX-189 against wild type and resistant mutant HCV replicons.

6.3.1 Anti-HCV activity of INX-189 against genotype 1a, 1b and 2a replicon cells.

Further *in vitro* evaluation was carried out in comparative replicon assays (Inhibitex Inc.), using replicon genotypes 1a, 1b and 2a. **INX-189** was found to be a very potent inhibitor of all tested HCV genotypes (Table 6.3).

| HCV genotype | EC ₅₀ [μM] | EC ₉₀ [μM] | CC ₅₀ [μM] for Huh7 |
|--------------|-----------------------|-----------------------|--------------------------------|
| 1a | 0.012 ± 0.004 | 0.042 ± 0.011 | 7.01 ± 1.97 |
| 1b | 0.010 ± 0.006 | 0.038 ± 0.020 | 7.01 ± 1.97 |
| 2a | 0.0009 ± 0.0001 | 0.0065 ± 0.001 | 7.01 ± 1.97 |

Table 6.3. INX-189 potency in wild type replicons.

During 72 h of exposure, the EC₅₀ concentration values for genotype 1a were ranged from 0.008-0.016 μM (mean 0.012 μM), for genotype 1b: 0.004-0.016 μM (mean 0.010 μM) and for genotype 2a: 0.0008-0.0010 μM (mean 0.0009 μM). For all tested genotypes INX-189 maintain its potency in the low nanomolar range.

To fully define the potency of **INX-189**, multiple HCV replicon 1b assays were performed. The compound was tested in 16-replicates and results are shown in Figure 6.3.

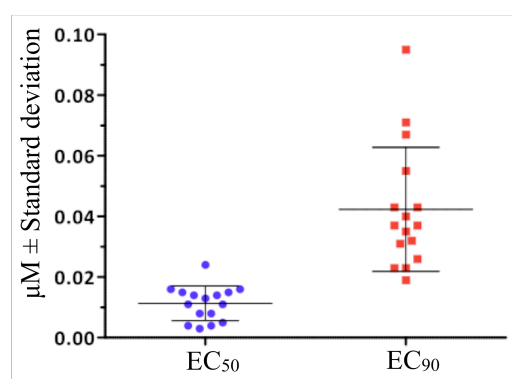


Figure 6.3. Potency of **INX-189** in the HCV genotype 1b replicon assay. Data represent results obtained from 16 replicates.

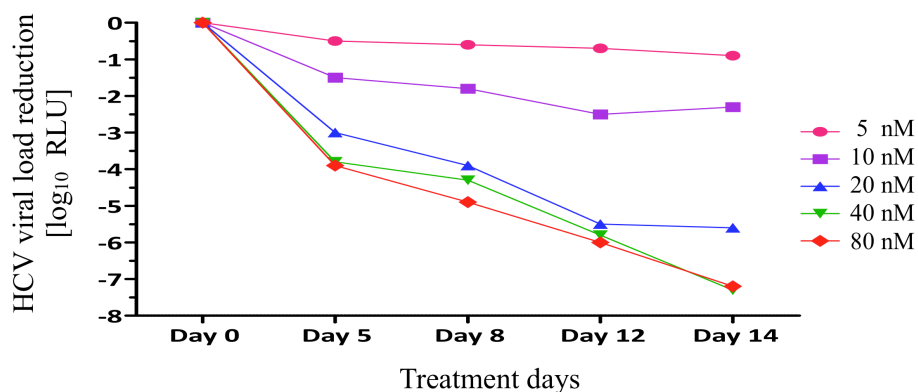
As shown in Figure 6.3, EC_{50} and EC_{90} values were ranging from 0.003-0.024 μM and 0.019-0.095 μM , respectively. These data revealed that **INX-189** is one of the most potent nucleoside based inhibitors of HCV polymerase reported up to date. The summary of the replicon activities of **INX-189** competitors is reported in Table 6.4.

| Cpd | Name | Genotype 1b EC_{90} [μM] |
|------|----------|---|
| 2.7 | RG7128 | 4.6 |
| 2.13 | PSI-7977 | 0.42 |
| 2.14 | IDX184 | 0.4 (EC_{50}) |
| 2.15 | INX-189 | 0.038 |
| 2.16 | PSI-938 | 1.38 |

EC_{90} : 90% effective concentration or compound concentration required to inhibit HCV replication by 90%;
Table 6.4. Summary of the replicon 1b activities of **INX-189** competitors.²

6.3.2 Rebound and clearance studies of **INX-189**.

In the next step, **INX-189** was evaluated by Inhibitex Inc. in a long-term treatment of HCV genotype 1b replicon cells. Cells were incubated in the presence of increasing concentrations of **INX-189** (0- control, 5, 10, 20, 40 and 80 nM) for 14 days. At specified time points (5, 8, 10, 12 and 14-days) cells were harvested and replication activity was measured as a HCV viral load reduction based on Renilla luciferase gene expression. Corresponding data are reported in the Figure 6.4.



RLU –Relative luminescence unit.

Figure 6.4. *In vitro* clearance of wild-type HCV replicon 1b with increasing concentrations of **INX-189**.

As reported in Figure 6.4, treatment of the replicon cells with **INX-189** resulted in decrease of viral load over time comparing with the control data. When cells were incubated with concentrations of **INX-189** as low as 5 nM ca. 1 \log_{10} reduction of viral load was observed. Incubation with 10 nM of compound, gave more than 2 \log_{10} decrease in HCV replication activity at day 14. Culturing in the presence of 20 nM solution of **INX-189** resulted in $>5 \log_{10}$ decline in viral load at day 12. When cells were incubated in the presence of 40 nM concentration of **INX-189**, which corresponds to the EC_{90} - concentration required to eradicate 90% of infected cells, HCV replication activity was reduced nearly to the baseline. Not much difference was observed when concentration of **INX-189** was increased to 80 nM (2x EC_{90}) comparing to the 40 nM data.

In general inhibition of HCV replication was found to be dose dependent up to 40 nM.

Additionally at days 5, 10 and 14 treated cells were harvested and subcultured without **INX-189** but in the presence of G418- specific antibiotic used for the selection of neomycin resistant colonies. Any cells that were remaining in the treated cultures and were able to express replicon genome would confer resistance to the G418 treatment, as the replicon genome contains neomycin resistance gene (Chapter 4). As these secondary cultures grew, individual flasks were fixed and stained with crystal violet (Figure 6.5). For the colonies where there was no visible growth, flasks were stained after 5-weeks.

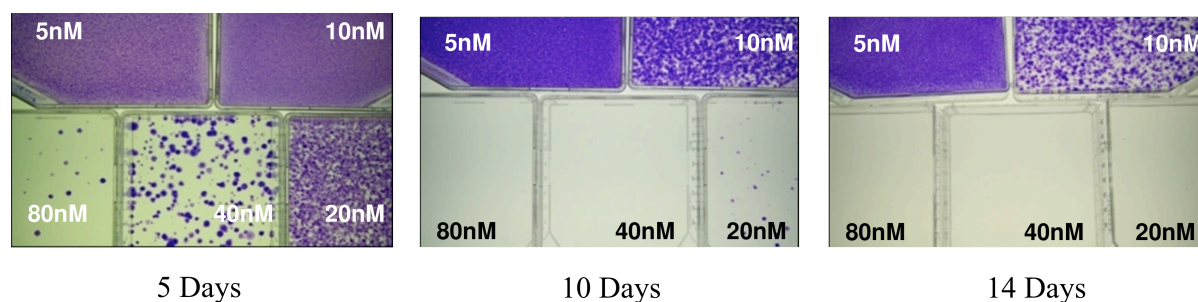


Figure 6.5. *In vitro* clearance of wild-type HCV replicon 1b with increasing concentrations of **INX-189** under G418 selection.

As can be seen in Figure 6.5, after 5 days of treatment all treated colonies retain their replicating ability regardless the concentrations, although the replication fitness diminished with increased concentration of **INX-189**. After 10 days of culturing, complete eradication of replicon expressing cells was achieved with 80 nM (2x EC₉₀) and 40 nM (1x EC₉₀) concentrations. During 14-days treatment, complete elimination of replicon expressing cells was attained at concentration as low as 20 nM. The ability of **INX-189** to clear the replicon and prevent the cells from recovering their replication ability at such a low concentration (~2x EC₅₀) is striking. In contrast **IDX184** (2.14) another 2'-C-methylguanosine monophosphate prodrug required a concentration of ≥ 2.5 μ M to achieve complete clearance of replicon expressing cells.³

6.3.3 Activity of **INX-189** against resistant mutant strains.

Emergence of resistance has become a concern for antiviral compounds because it may lead to viral breakthrough and treatment failure. In order to evaluate the ability of **INX-189** to select for resistance, selection studies were performed using genotypes 1b and 1a. All studies were performed at Inhibitex Inc.

Replicon cells were cultured in the presence of increasing concentrations of **INX-189** up to eight times EC₅₀ (80 nM) in the 1b replicon and up to 40 nM (4x EC₅₀) in the 1a replicon. All colonies were incubated in the presence of G418 antibiotic. Emergence of resistance was monitored at various time points. The establishment of stable **INX-189** escape mutants required 6 to 9-weeks.

During selection studies, resistant colonies were isolated and the nucleotide sequence of the NS5B genes were determined. Two consistent NS5B mutations were found. In the first

mutation serine 282 codon was replaced by threonine (S282T). This particular S282T mutation has been previously identified as a resistant mutant for 2'-C-methylguanosine TP.⁴ A second change was alteration of the amino acid in position 585 from isoleucine to threonine (I585T). Selection studies carried out in clone 1a resulted in one single mutation in position 540; alanine codon was replaced by threonine (A540T). To determine whether these amino acid alterations could be sufficient to confer resistance to **INX-189** and if they can have effect on replication fitness, replicon and transient assays were performed. Additionally activity of **INX-189** against a replicon carrying resistance to 4'-azidocytidine **2.4** (S96T and N142T) was evaluated alongside. All data are reported in Table 6.5.

| NS5B mutation | replication fitness (% wild-type) | EC ₅₀ of INX-189 [μM] |
|---------------|--------------------------------------|----------------------------------|
| Wild-type | 100 | 0.006 ± 0.003 |
| S282T | 4 | 0.074 ± 0.026 |
| I585T | 165 | 0.005 ± 0.001 |
| A540T | 86 | 0.004 ± 0.001 |
| S282T/I585T | 8 | 0.055 ± 0.018 |
| S96T/N142T | 102 | 0.006 ± 0.001 |

Table 6.5. INX-189 Potency against wild type and transient replicons expressing mutant NS5B.

Results of the fitness analysis showed that S282T replicon was the most unfit replicon (4% of wild-type) followed by the replicon carrying double mutation S282T/I585T (8%), comparing to the wild-type. What is striking is that the I585T mutation increased the replication efficiency comparing to the wild-type replicon, and most probably this mutation is responsible for improved replication fitness of S282T/I585T in comparison with S282T replicon. The potency of **INX-189** across all mutant strains was retained, with the only exception of mutants carrying S282T alteration. In these cases the EC₅₀ values were reduced approximately by 10-fold. Regardless of loss of activity by ca. 10-fold, INX-189 could still efficiently inhibit HCV replication in S282T mutant replicons with an EC₉₀ values of 0.34 ± 0.17 μM (Figure 6.6).

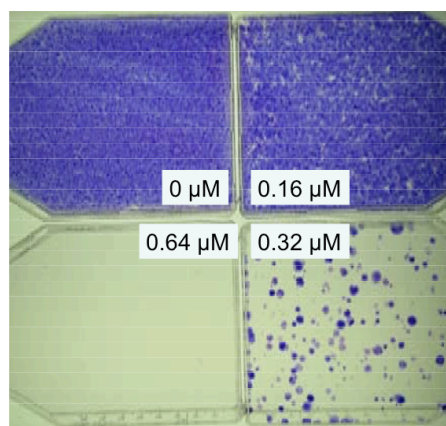


Figure 6.6. *In vitro* clearance of HCV 1b S282T stable replicon with increasing concentrations of INX-189.

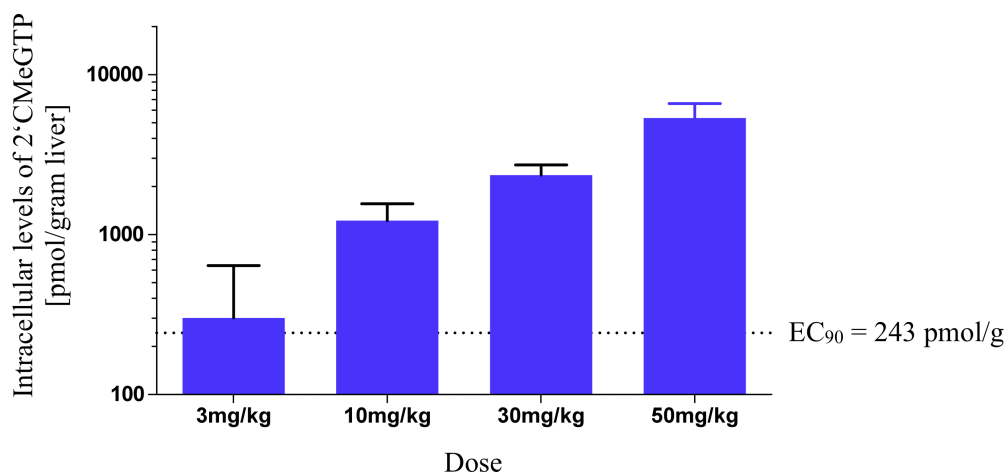
6.4 Intracellular levels of 2'-C- β -methylguanosine triphosphate – *in vivo* studies.

Initial studies carried out in replicon 1b cells and primary human hepatocytes revealed excellent ability of **INX-189** to produce high levels of intracellular 2'CMeG 5'-triphosphate. Subsequently, the ability of **INX-189** to deliver monophosphate inside the liver cells and subsequent formation of 2'CMeGTP after oral administration was investigated in different animal models. All studies were performed at Inhibitex Inc.

6.4.1 Intracellular levels of 2'-C- β -methylguanosine triphosphate in rat model.

INX-189 was evaluated in rat model (Sprague-Dawley rats) to investigate metabolism and pharmacokinetics after oral administration. The compound was formulated in 95% Capmul MCM and 5% Tween 80, and administered as oral gavage to male Sprague-Dawley rats.

Rats were orally treated with an ascending dose of **INX-189**, starting from 3 mg/kg up to 50 mg/kg of body weight, once daily. After 24 h of treatment, liver samples were harvested and levels of 2'-C-methylguanosine TP were analysed using LC-MS/MS tandem spectroscopy (Figure 6.7).



EC₉₀: 90% effective concentration or compound concentration required to inhibit HCV replication by 90%;

Figure 6.7. Concentrations of 2'-CMeGTP in rat liver following single oral dose of **INX-189**.

As reported in Figure 6.7, 24 h post dose, at all tested concentrations, levels of 2'-C-methylguanosine triphosphate in the rat liver exceeded the EC₉₀ values. It can be seen that with the dose as low as 10 mg/kg (97 mg HED) **INX-189** was able to produce ca. 2200 pmol of intracellular triphosphate per gram of liver tissue.

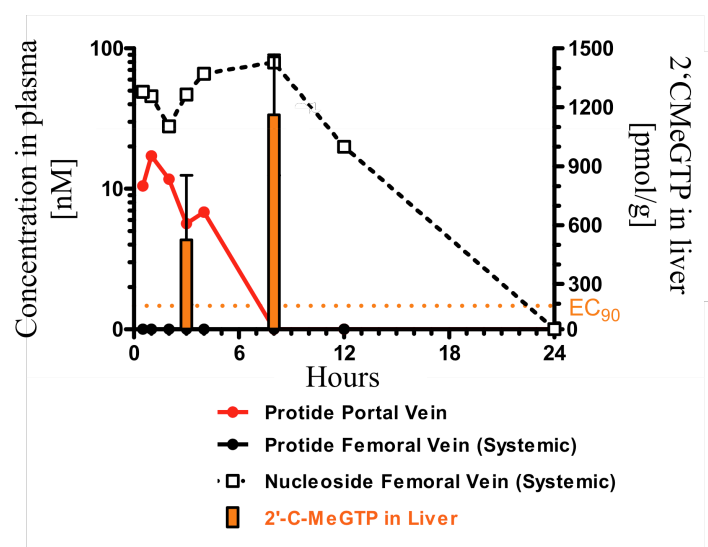
During prolonged (7 days) studies, it was observed that liver concentrations of 2'-CMeG 5'-triphosphate exceeded the EC₉₀ soon after dosing, and remained at or above this level for 72 h and decayed slowly after that time.

6.4.2 Intracellular levels of 2'-C-β-methylguanosine triphosphate in cynomolgus monkey model.

In order to investigate the ability of **INX-189**, to generate sufficient levels of 2'-C-methylguanosine triphosphate in the liver of cynomolgus monkey, four animals were orally dosed with **INX-189** at 25 mg/kg (equivalent of 50 mg/kg in rats). The compound was formulated in 5% DMA, 20% Solutol HS, 15-20% PEG 400, 55-60% 50mM sodium acetate, pH 4.0, and was administrated as a single oral gavage dose.

All non-human primate studies were conducted at MPI Research Inc.

Liver biopsy samples (four to five samples per animal) were collected at 3 h and 8 h post-dose. Blood/plasma samples were collected from the portal and femoral veins (0.5, 1, 2, 3, 4, 8, 12 and 24 h post administration) to determined levels of **INX-189** being absorbed from the gastrointestinal tract and to measure the systemic exposure to **INX-189** and 2'-CMeG nucleoside (Figure 6.8).



EC₉₀: Defined as the amount of intra cellular triphosphate necessary to achieve 90% inhibition in the HCV replicon assay.

Figure 6.8. Oral pharmacokinetics in nonhuman primates following single oral dose of **INX-189**.

According to the data shown in Figure 6.8, the concentration of intact **INX-189** was measured in portal vein plasma shortly after oral administration (0.5 h). The highest concentration of **INX-189** was achieved within 1 h and remained detectable for a short period of time, approximately 6 - 8 h. **INX-189** was not detected in systemic circulation (femoral vein samples) at any time point. These data clearly suggest that at this concentration of compound (25 mg/kg), **INX-189** was efficiently extracted by the liver.

As reported in Figure 6.8, the mean concentration of the 2'-C-methylguanosine triphosphate, at 3 h post administration, was 525 ± 233 pmol/g liver (282 ± 177 ng/g) and increased over time (8 h) to 1160 ± 216 pmol/g liver (624 ± 165 ng/g). Comparing to the *in vitro* data (EC₉₀ = 234 pmol/g liver, 130.5 ng/g), the EC₉₀ was exceeded by 2.2- and 4.95-fold at the respective time points. Since these data represent a triphosphate levels obtained after a single dose treatment, multiple dosing would be expected to significantly improve triphosphate liver exposure.

As the measurement of the 2'CMeGTP as an analytical tool is highly limited, the levels of 2'-C-methylguanosine nucleoside were measured in femoral vein. 2'CMeG is one of the metabolites of **INX-189** and can be used as a surrogate analyte of 2'CMeGTP to monitor pharmacokinetics in HCV patients. The cynomolgus monkey data indicate a linear correlation between 2'CMeG AUC₂₄ values and AUC₂₄ of 2'CMeGTP.

All presented data supported the progression of **INX-189** into Phase 1a clinical development.

6.5 Results of the Phase Ia clinical trials of INX-189.

The aim of the Phase Ia clinical trials was to evaluate the safety profile, tolerability and pharmacokinetics in healthy volunteers. Some 42 healthy subjects were enrolled for the trial and were evaluated in a single ascending dose, double blind, placebo controlled study. During the treatments recipients were orally administered with dose ranging from 3 mg to 100 mg of **INX-189**.

In general **INX-189** was well-tolerated at all tested dose levels. During the trial, no drug-related serious adverse events, frequency or type of adverse events were observed. The occurring side events were no different than the ones seen in the placebo group. The most common events, which had occurred in more than one subject, were headaches and nasal congestions.

Pharmacokinetic data support once a day, oral dosing.

Based on all available data, **INX-189** was further progressed into Phase Ib clinical development.

6.6 Results of the Phase Ib clinical trials of INX-189.

The Phase Ib trial was carried out on HCV genotype 1 treatment-naïve patients, who where received an all-oral regimen of **INX-189** and/or RBV against placebo-controlled group. 80 Patients were divided into 8 cohorts and randomized 8:2, to receive either **INX-189** in a dose ranging from 9 mg to 100 mg or placebo, once daily for 7-days (Figure 6.9a). One cohort was dosed 50 mg on day 1, followed by 9 mg of **INX-189** or placebo on days 2-6. Additional two cohorts were treated with **INX-189** (9 mg or 25 mg) in combination with RBV for 7 days (Figure 6.9b). All groups were monitored up to 14 days.

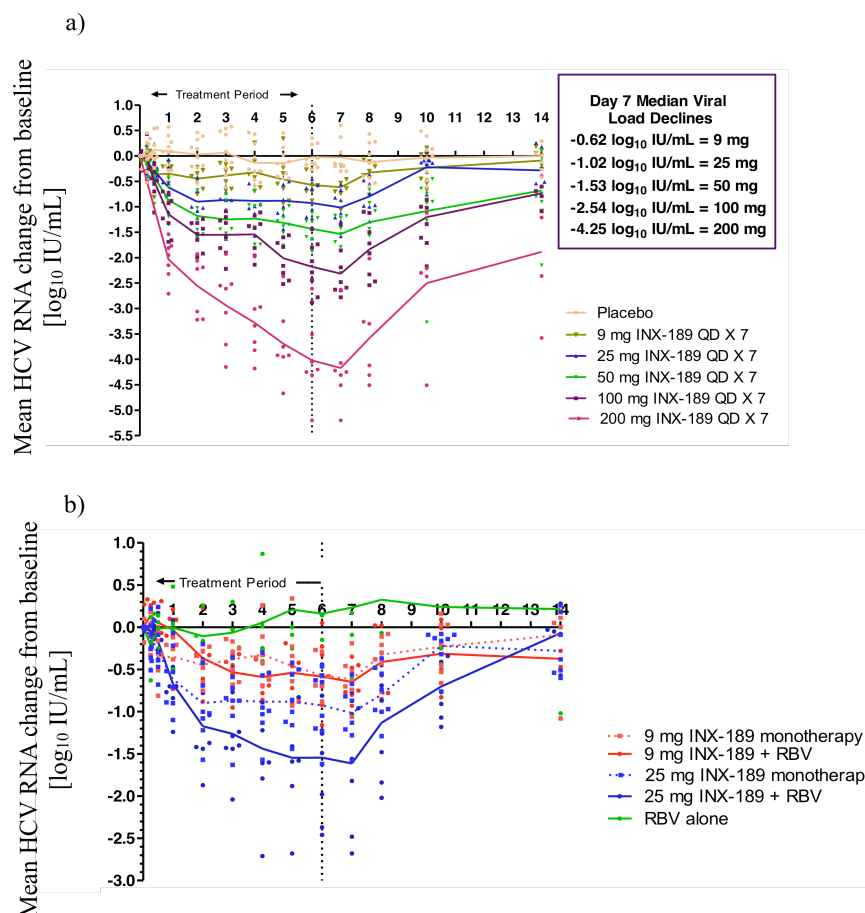


Figure 6.9. Median HCV RNA changes from baseline. a) During treatment with ascending dose of **INX-189**; b) **INX-189** + RBV combination study.

All tested doses of **INX-189** resulted in significant decrease of viral replication, based on the reduction of HCV RNA levels from baseline. As summarized in Figure 6.9, **INX-189** monotherapy at 100 mg QD for seven days, provided mean 2.54 log₁₀ IU/mL decrease in viral RNA, while 200 mg QD produced a highly significant median 4.25 log₁₀ IU/mL reduction in HCV viral load. In general **INX-189** demonstrated dose dependent effects.

When ribavirin (RBV) was added to the 25 mg of **INX-189**, the average drop in HCV RNA was ca. 0.5-log greater than monotherapy. This data indicate that **INX-189**, when dosed once daily at 9 mg or 25 mg in combination with ribavirin demonstrates dose-dependent, synergistic antiviral effect.

Additional data from Phase Ib clinical trials revealed that **INX-189** was generally well tolerated at all tested doses. There were no significant adverse events related to the drug. Reported adverse events were mild or moderate in severity, with headache being the most common one. In the 25 mg **INX-189** + RBV group there was one case of grade 3 laboratory

abnormality (decrease in hemoglobin level). There were no discontinuations of treatment due to adverse events.

Upon successful completion of Phase Ib clinical trials, **INX-189** entered Phase II clinical development in August 2011.

References:

1. http://files.shareholder.com/downloads/VRUS/0x0x345285/1de90b6b-1e21-4a23-b59f-f4533817aa73/PSI-7977%20slides_PR012110.pdf
2. http://regist2.virology-education.com/5thHepC/docs/13_McCarville.pdf
3. Chamberlain S. INX-08189 A novel double pro-drug and clinical candidate for hepatitis C virus. *The 10th Winter Conference on Medicinal and Bioorganic Chemistry*, 23rd-27th January 2011, Colorado, USA.
4. Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, Cortese R, De Francesco R, Eldrup AB, Getty KL, Hou XS, LaFemina RL, Ludmerer SW, MacCoss M, McMasters DR, Stahlhut MW, Olsen DB, Hazuda DJ, Flores OA. Characterisation of resistance to non-obligate chain-terminating ribonucleosides analogues that inhibit hepatitis C virus replication in vitro. *J Biol Chem* **2003**, 278, 49164-49170.

CHAPTER SEVEN

New ProTide approach: Cyclic phosphoramidate (oxazaphosphorine) based prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine.**7.1 Cyclic phosphoramidates – rational behind the design.**

As already mentioned in previous Chapters, all nucleoside analogues require kinase dependent activation to their corresponding 5'-monophosphates in order to be active. Considering the fact that usually the first phosphorylation step is recognized as a rate limiting¹, we decided to investigate new pro-nucleotide approach, in order to deliver 5'-monophosphates intracellularly.

We decided to focus our attention on cyclic phosphoramidate prodrugs. It is known in the literature that oxazaphosphorine based prodrugs (e.g. cyclophosphamide, Scheme 7.1) can undergo metabolic activation by hepatic P₄₅₀-dependent mixed-function oxidases to give the biologically active agent (phosphoramidate mustard, Figure 7.1).²

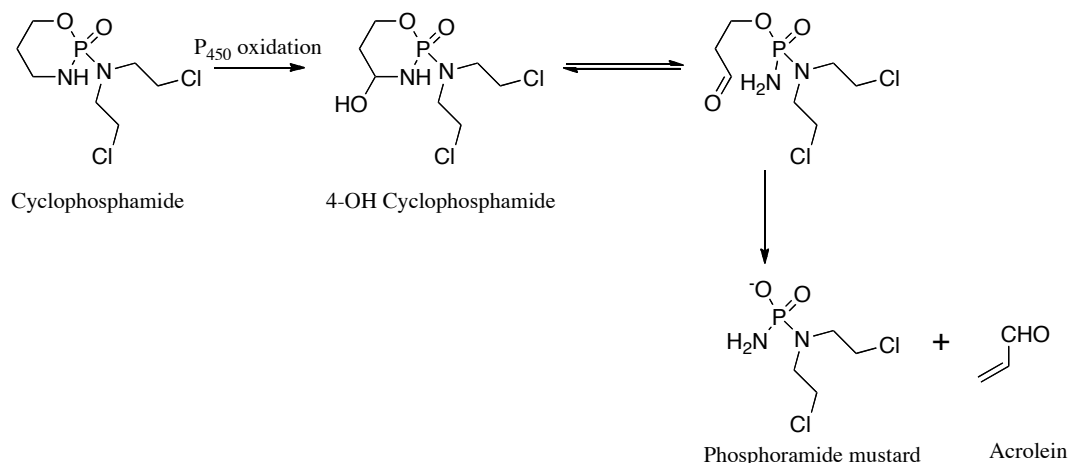


Figure 7.1. Metabolic activation of cyclophosphamide.

As this strategy depends on cytochrome P₄₅₀ (highly expressed in the liver) catalyzed cleavage, the introduction of oxazaphosphorine moiety as a 5'-monophosphate masking unit, could result in liver specific prodrugs with more potent activity, lower systemic exposure and consequently lower toxicity.

In this Chapter, an effort to develop a series of oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine, with general structure shown in Figure 7.2, will be reported.

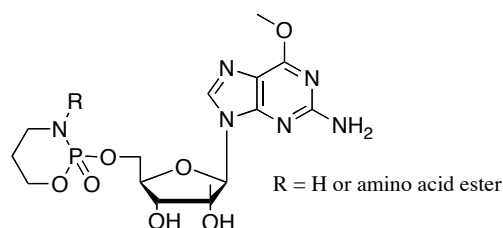


Figure 7.2. General structure of cyclic phosphonamide prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine.

7.2 Synthesis of phosphorylating reagents.

In order to synthesise the family of oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine, the appropriate phosphorylating agents had to be prepared. Our interest was focused on the 2-chloro-1,3,2-oxazaphosphinane 2-oxide **7.1** and amino acid derived cyclic phosphorochloridates **7.2a-c** (Figure 7.3).

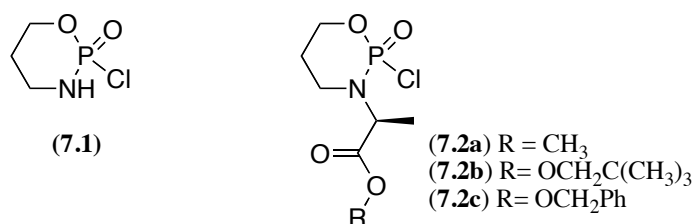
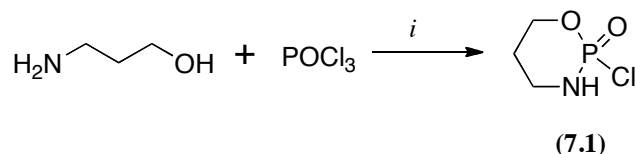


Figure 7.3. Chemical structures of phosphorylating agents **7.1** and **7.2a-c**.

7.2.1 Synthesis of 2-Chloro-1,3,2-oxazaphosphinane 2-oxide.

To prepare **7.1**, phosphorus oxychloride in dry chloroform (CHCl_3) was cooled down to $-15\text{ }^\circ\text{C}$. A solution of 3-aminopropanol and Et_3N in dry CHCl_3 was added dropwise with stirring to the reaction mixture. Reaction was kept at $-10\text{ }^\circ\text{C}$ and additional portion of Et_3N in CHCl_3 was added. The reaction mixture was maintained at $0\text{ }^\circ\text{C}$ for 18 h. After that time, solvents were removed under reduced pressure (at temperature not higher than $35\text{ }^\circ\text{C}$).³ The solid residue was extracted with dry acetone and evaporated to dryness. The crude mixture was purified on silica gel using ethyl acetate : hexane (1:1) as an eluent and pure product was obtained as a white solid in 84% yield (Scheme 7.1).



Reagents and Conditions: i) Et₃N, CHCl₃, -15 °C to 0 °C, 18 h.

Scheme 7.1. Synthesis of 2-chloro-1,3,2-oxazaphosphinane 2-oxide.

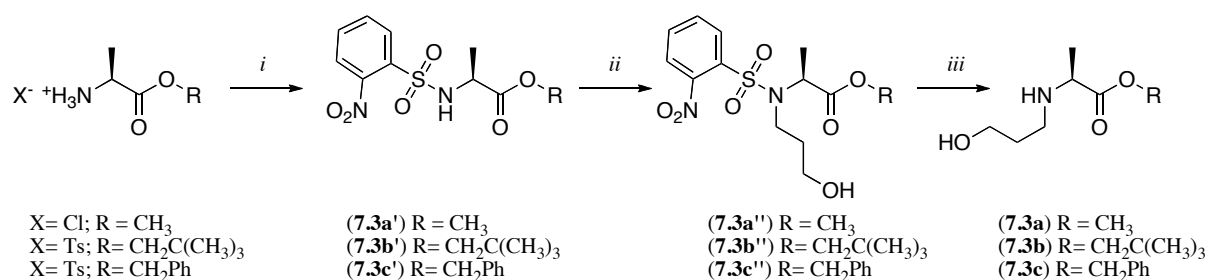
7.2.2. Synthesis of amino acid derived cyclic phosphorochloridates.

To synthesise the amino acid derived cyclic phosphonamides, firstly *N*-alkylation of the α-amino group of precursor amino acids was investigated.

There are a number of different *N*-alkylation methods of α-amino acids reported in the literature, however none of them is general, and without problems. The most common method is the alkylation of sulfonamides (usually tosylates) of α-amino acids. Nevertheless this method was found to be problematic due to the difficulties in tosylate protecting group removal.⁴ Fukuyama and co-workers reported facile synthesis of mono *N*-alkylated α-amino acids using 2-nitrophenylsulphonamide derivatives.⁵ We decided to use this protocol in order to obtain a family of *N*-hydroxypropyl α-amino acid esters, which could be subsequently used for the synthesis of cyclic phosphorochloridates.

For the synthesis, enantiomerically pure L-alanine amino acid was chosen. L-alanine methyl ester hydrochloride and L-alanine benzyl ester tosylate salt were commercially available; L-alanine 2,2-dimethylpropyl ester tosylate salt was prepared according to the procedure reported in Chapter 3.

In the first instance, the amino acid esters salts were protected with a 2-nitrobenzenesulfonyl group (Scheme 7.2). The appropriate amino acid ester was dissolved in dry dichloromethane and Et₃N was added dropwise to the stirring solution. The reaction mixture was cooled down in an ice/H₂O bath and 2-nitrosulfonylbenzyl chloride was added. After that, reaction mixture was allowed to warm to ambient temperature and the reaction was stirred overnight.⁵ The resulting solution was washed 3x with water, dried over anhydrous Na₂SO₄ and concentrated. The resultant *N*-(2-nitrophenylsulfonyl)-L-alanine esters **7.3a'-c'** were obtained as yellowish oils and were used for the subsequent step without any purifications. Compounds were obtained in quantitative yields.



Reagents and Conditions: i) 2-nitrobenzenesulfonyl chloride, Et_3N , CH_2Cl_2 , $-5\text{ }^\circ\text{C}$ to rt, overnight; ii) Cs_2CO_3 , 3-bromopropanol, DMF, $60\text{ }^\circ\text{C}$, overnight; iii) K_2CO_3 , PhSH, ACN, rt, overnight.

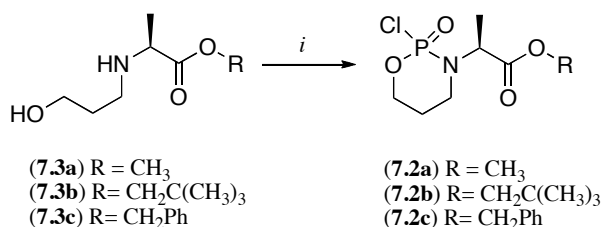
Scheme 7.2. Synthesis of *N*-alkylated amino acid esters **7.3a-c**.

Alkylation of 2-nitrophenylsulfonamides **7.3a'-c'** was performed in the presence of cesium carbonate in dry dimethylformamide (Scheme 7.2). The reaction mixture was heated to $60\text{ }^\circ\text{C}$ and 3-bromopropanol was added dropwise with stirring.^{5,6} The resulting solution was heated overnight and after that time was diluted with water and extracted 3x with ethyl acetate. The combined organic phases were washed with 3M calcium chloride solution, water and brine. After that, the solution was dried over anhydrous Na_2SO_4 and solvent was evaporated. The crude mixture was purified on silica gel using light petroleum : ethyl acetate (8:2) as an eluent, to afford compounds **7.3a''-c''** in moderate yields (25-31%). These yields were somewhat lower than those reported for the alkylation of simple amines.⁵ It can be explained by the fact that the presence of the α -ester causes an extra electron withdrawing effect and as a consequence the anion of nitrophenylsulfonamide is less nucleophilic, and therefore reacts relatively slow with 3-bromopropanol. As the amounts of the *N*-alkylated intermediates were sufficient to progress with the synthesis, optimization of the reaction conditions was not investigated.

The removal of the protecting 2-nitrophenylsulfonyl group was accomplished using thiophenol in dry acetonitrile.⁶ After reaction completion, the reaction mixture was concentrated in vacuum and the residue was taken up in diethyl ether. 1M Hydrochloric acid was added to the resulting mixture and stirred for 10 min. After that time, the organic layer was removed and washed with water. The combined aqueous layers were washed with diethyl ether, followed by basification with solid K_2CO_3 . The product was extracted from the basic aqueous phase with ethyl acetate. Combined organic phases were dried over Na_2SO_4 , evaporated and purified on silica gel, using $CHCl_3/MeOH$ (9:1) as an eluent to afford the

substituted free amino alcohols **7.3a-c** (Scheme 7.2). Pure compounds were obtained as a yellowish oils, in reasonable yields (42-67%).

Synthesised *N*-alkylated amino acid esters were used for the cyclisation reaction in the presence of POCl₃ and Et₃N (Scheme 7.3).



Reagents and Conditions: i) POCl₃, Et₃N, CHCl₃, -15 °C to 0 °C, 18 h.

Scheme 7.3. Synthesis of amino acid derived cyclic phosphorochloridates **7.2a-c**.

After reaction completion, the solvent was removed under reduced pressure at 35 °C. The crude mixture was purified on silica gel using Hexane/AcOEt (7:3) as an eluent. All compounds were obtained as clear, colourless oils in good to excellent yields (Table 7.1).

| Cpd | AA | R | ³¹ P NMR [δ ppm] | Isomer ratio | Yield % |
|-------------|-------|--|-----------------------------|--------------|---------|
| 7.1 | - | - | 9.22 | - | 84 |
| 7.2a | L-Ala | CH ₃ | 9.27, 8.62 | 53:47 | 87 |
| 7.2b | L-Ala | CH ₂ C(CH ₃) ₃ | 9.04, 8.62 | 57:43 | 75 |
| 7.2c | L-Ala | CH ₂ Ph | 9.15, 8.61 | 52:48 | 90 |

Table 7.1. Summary of synthesised cyclic phosphorochloridates **7.1** and **7.3a-c**.

All synthesised compounds were obtained as roughly equimolar mixtures of diastereoisomers at phosphorus centre (based on the ³¹P NMR). In the case of **7.1** overlapping of the enantiotopic ³¹P NMR signals was presumably observed (Table 7.1).

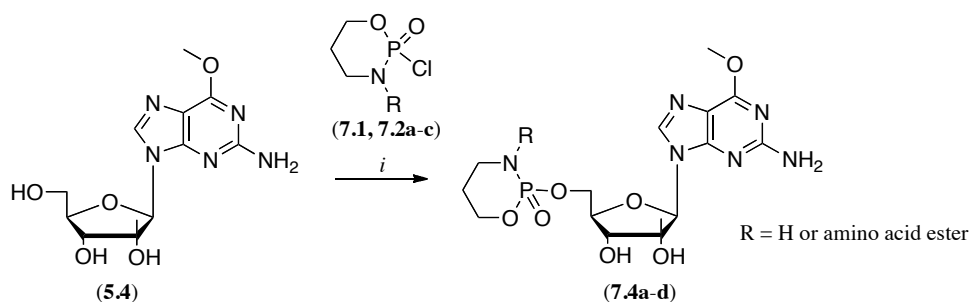
The synthesised compounds **7.1** and **7.3a-c**, were used for the coupling reaction with 6-*O*-methyl-2'-*C*-methyguanosine.

7.3 Synthesis of oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine.

In order to synthesise the family of oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine, appropriate coupling conditions had to be investigated.

In the first instance *t*BuMgCl-mediated coupling was considered. Application of the same procedure as used for the synthesis of a large family of 6-*O*-methyl-2'-*C*-methylguanosine ProTides (described in Chapter 5), resulted in a complex mixture of products, which proved to be very difficult to purify.

To avoid the formation of unwanted side products, different coupling procedures were investigated. The most successful one was a simple coupling reaction in the presence of pyridine as a sole solvent/reagent.³ Compounds **7.4a-d** were synthesised according to the following procedure: To a mixture of 6-*O*-methyl-2'-*C*-methylguanosine and appropriate cyclic phosphorochloridate under argon atmosphere, dry pyridine was added and reaction mixture was stirred for 20 h at ambient temperature (Scheme 7.4).

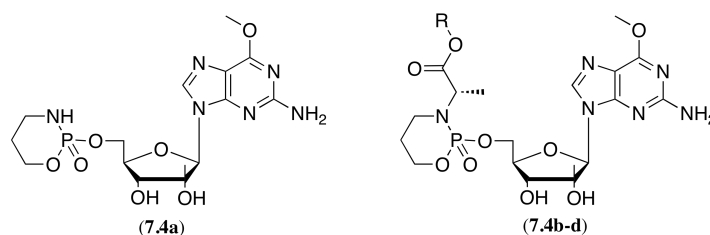


Reagents and Conditions: i) Pyridine, rt, 20 h.

Scheme 7.3. Synthesis cyclic phosphonamide prodrugs **7.4a-d**.

After that time, pyridine was removed under reduced pressure followed by co-evaporation with toluene and MeOH. In case of **7.4a**, the residue was purified on silica gel using CHCl₃ : EtOH (0 to 5% gradient) as an eluent. Use of ethanol provided better product separation than when more polar MeOH was used. For compounds **7.4b-c**, chromatographic purification was performed using CHCl₃/MeOH (9:1) as an eluent.

All pure compounds were obtained as white solids in low yields 3-16% (Table 7.2).



| Cpd | AA | R | ^{31}P NMR [δ ppm] | Yield% | Isomer ratio |
|-------------|-------|--|-------------------------------------|--------|--------------|
| 7.4a | - | - | 5.48, 5.44 | 16 | 58:42 |
| 7.4b | L-Ala | CH ₃ | 4.62, 4.48 | 11 | 93:7 |
| 7.4c | L-Ala | CH ₂ C(CH ₃) ₃ | 4.46, 4.05 | 3 | 91:9 |
| 7.4d | L-Ala | CH ₂ Ph | 4.46, 4.07 | 6 | 95:5 |

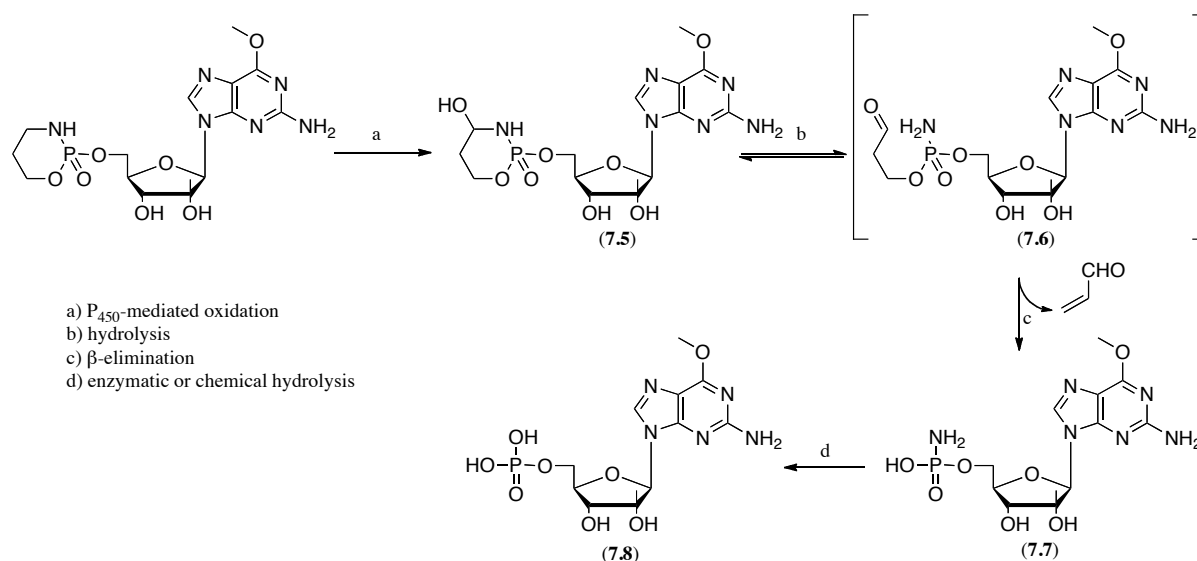
Table 7.2. Summary of synthesised cyclic phosphonamide ProTides **7.4a-d**.

Interestingly only **7.4a** was isolated as roughly 1:1 mixture of phosphorus diastereoisomers (Table 7.2). After NMR and HPLC analysis of isolated prodrugs **7.4b-d**, it was found that one of the isomers was greatly predominant comparing to the other one. In all cases, main/major isomer peak had more downfield NMR shift. It is clear that during the reaction strong stereoselective preference was observed. It has to be noted, that cyclic phosphorochloridates used for the coupling reactions were represented by circa equimolar mixtures of diastereoisomers (Table 7.1).

These findings may be useful if stereoselective synthesis of phosphoramidates was of interest.

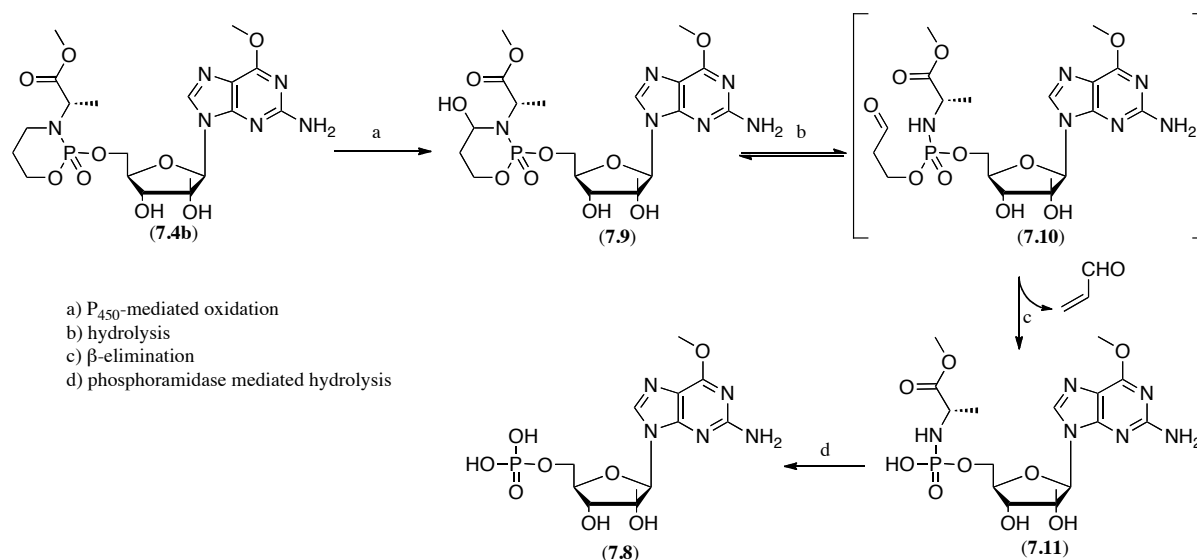
7.4 Putative metabolic activation pathway.

The suggested mechanism of activation of the cyclic phosphonamide prodrug of 6-*O*-methyl-2'-*C*-methylguanosine **7.4a** involves oxidation of carbon adjacent to the nitrogen atom (Scheme 7.4). Subsequent hydrolysis of **7.5** afforded the second intermediate **7.6**, which can undergo facile β -elimination to give intermediate **7.7** and acrolein. It was postulated that conversion of phosphoramidate **7.7** to the corresponding 5'-monophosphate **7.8** would occur by chemical or enzymatic hydrolysis (Scheme 7.4). Released monophosphate could be then deaminated by specific deaminases to give 2'-*C*-methylguanosine 5'-monophosphate, which could be further phosphorylated to the active triphosphate species.



Scheme 7.4. Suggested mechanism of activation of oxazaphosphorine prodrug **7.4a**.

In the case of the amino acid derived analogues **7.4b-d**, a similar route would be expected. Based on the work of Lu *et al.*⁷ the activation process would start with the oxidation of the carbon adjacent to the amide. The following activation pathway would lead to the formation of 5'-monophosphate intermediate **7.8** (Scheme 7.5). However it is not clear, whether the ester moiety would be cleaved or not, at any stage of the activation process. It is possible that the phosphoramidase type enzyme, responsible for the P-N bond cleavage, could perform the deamination reaction on the amino acid ester, as long as the ester moiety does not cause steric hindrance in the active site of the enzyme.



Scheme 7.5. Suggested mechanism of activation of oxazaphosphorine prodrug **7.4b**.

7.4.1 Human liver microsomes assay.

To probe the suggested mechanism of activation, compound **7.4a** was incubated in the presence of human liver microsomes (purchased from Sigma).⁸ Incubation was carried out without the presence of NADPH co-factor. The incubation mixture (final volume 600 μL) contained: 90 μL of microsomal protein, 235 μL of Tris buffer (pH 7.5) and **7.4a** (500 μM concentration in Tris buffer, 75 μL) and 200 μL of D_2O .

The reaction was followed by the ^{31}P NMR at 37 $^\circ\text{C}$, for 14 h. Spectra were recorded in 7 min intervals with blank spectra of compound **7.4a** in the Tris buffer (without microsomes) recorded first.

As can be seen in Figure 7.1, after addition of liver microsomes to the ProTide **7.4a** ($\delta_{\text{P}} = 7.08, 7.03 \text{ ppm}$) new species at $\delta_{\text{P}} = 2.24 \text{ ppm}$ started to form within the first 10-24 min. This new species, based on the ^{31}P NMR shift could correspond to the 6-*O*-methyl-2'-*C*-methylguanosine monophosphate **7.8**.

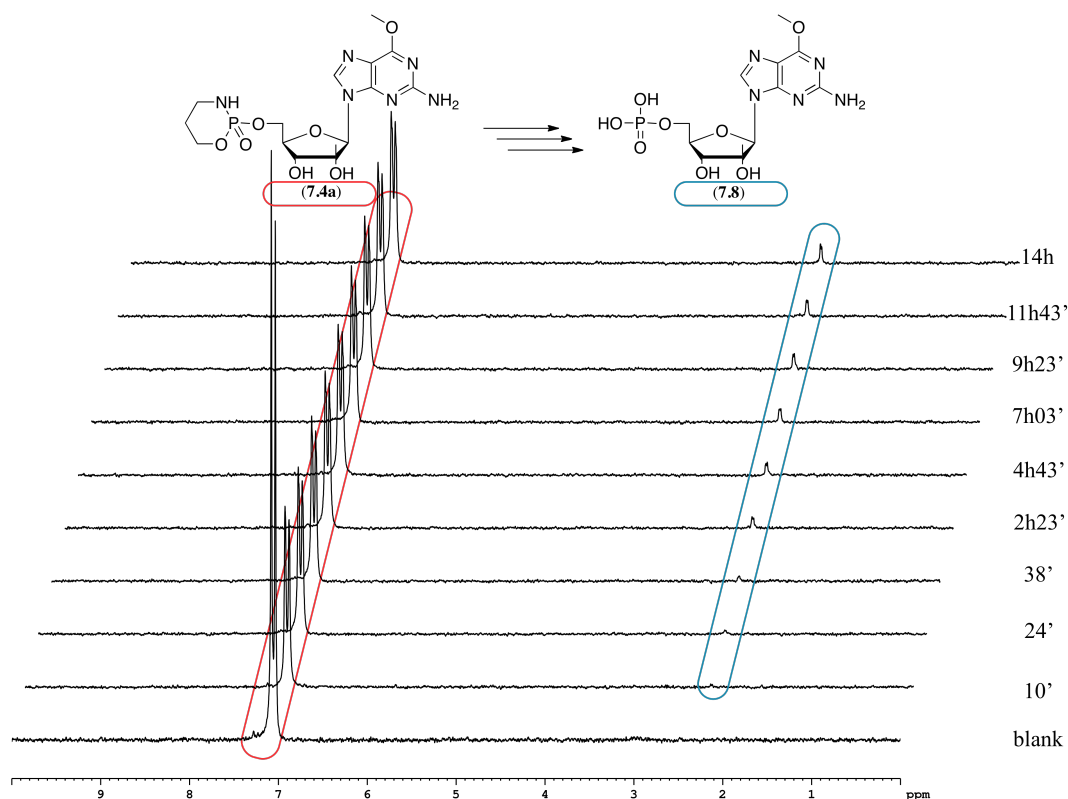


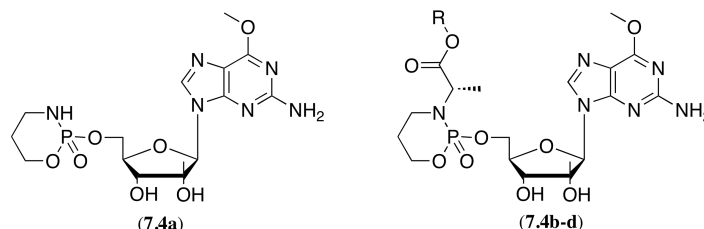
Figure 7.1. Formation of 5'-monophosphate **7.8** from oxazaphosphorine prodrug **7.4a** in liver microsomes, followed by ^{31}P NMR.

The processing rate for the compound **7.4a** was found to be very slow, with more than 90% of parent compound remaining after 14 h of incubation. It might be that the enzymes involved in the activation of cyclophosphamide were not fully active in this case or the six-membered cyclic phosphoramidate **7.4a** was too stable and therefore the release of monophosphate was not efficient. Any other ^{31}P NMR signals related to the metabolic intermediates **7.5-7.7** were not detected, suggesting that once the parent prodrug is oxidised subsequent steps leading to the monophosphate formation are very rapid. These findings can imply that the oxidation step is the rate-limiting step during the activation of oxazaphosphorine prodrugs.

To validate the results obtained from **7.4a**, additional assays on compounds **7.4b-d** should be performed. Furthermore, assays in the presence of NADPH as a co-factor need to be considered in order to fully understand the mechanism of activation.

7.5 Biological evaluation of cyclic phosphoramidate prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine.

All novel oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine (**7.4a-7.4d**) were evaluated by Inhibitex Inc., against hepatitis C virus *in vitro* using a replicon assay. Corresponding data are reported in Table 7.3.



| Cpd | AA | Ester (R) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|-------------|-------|--|-------|-----------------------|-----------------------|
| 5.4 | - | - | -0.6 | 4.6 | >100 |
| 7.4a | - | - | -1.1 | >40 | >100 |
| 7.4b | L-Ala | CH ₃ | 0.4 | >40 | >100 |
| 7.4c | L-Ala | CH ₂ C(CH ₃) ₃ | 2.3 | >10 | >100 |
| 7.4d | L-Ala | CH ₂ Ph | 2.1 | >10 | >100 |

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%;
 CC₅₀: 50% cytotoxic concentration.

Table 7.3. Anti-HCV activity and calculated lipophilicity of oxazaphosphorine prodrugs **7.4a-d**.

As can be seen in Table 7.3, none of the synthesized oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine exhibited anti-HCV activity. The lack of antiviral activity indicates that the 5'-monophosphate was not efficiently released from the tested oxazaphosphorine prodrugs **7.4a-d**. The explanation might be the fact that the Huh7 cell line (cell line used for the replicon assay) expresses low levels of CYP enzymes comparing to the primary human hepatocytes.⁹ This feature limits the utility of Huh7 cells for drug metabolism studies per se. As the cytochrome P₄₅₀-mediated oxidation of 1,3,2-oxazaphosphorine ring is essential for the activity of this class of compounds, decreased levels of cytochrome P₄₅₀ enzymes would result in deficiency of anti-HCV activity.

It is known that some of the drugs including rifampicin can induce CYP enzymes expression. It might be of interest to test compounds **7.4a-d** in replicon assay in the presence of cytochrome P₄₅₀ enzyme inducer.

7.6 Conclusions.

In summary, the synthesis and structure activity relationship of a novel class of oxazaphosphorine prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine was reported. All novel compounds were investigated for their ability to inhibit HCV replication *in vitro*. None of the newly synthesised compound exhibited anti-HCV activity in replicon assay. As an explanation lack of specific enzymes responsible for the oxidation of the oxazaphosphorine ring when tested in the replicon assay was suggested. To fully validate the potential of this class of molecules, further testing of these compounds in more appropriate conditions (presence of CYP inducing agents) is required.

References:

1. Hecker SJ, Erion MD. Prodrugs of phosphonates and phosphates. *J Med Chem* **2008**, 51, 2328-2345.
2. Cohen JL, Jao JY. Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat. *J Pharmacol Exp Ther* **1970**, 2, 206-210.
3. Hunston RN, Jehangir M, Jones AS, Walker RT. Synthesis of 2'-deoxy-5-fluoro-5'-O-1'', 3'', 2''-oxazaphosphacyclohexa-2''-yluridine 2''-oxide and related compounds. *Tetrahedron* **1980**, 36, 2337-2340.
4. Adlington RM, Mantell SJ. A radical route to 2(S)-4-exomethylene proline *Tetrahedron* **1992**, 48, 6529-6536.
5. Fukuyama T, Jow CK, Cheung M. 2- and 4-Nitrobenzenesulfonamides: Exceptionally versatile means for preparation of secondary amines and protection of amines. *Tetrahedron Lett* **1995**, 36, 6373-6374.
6. Cyclic phosphonamides and phosphinamides, novel series of potent matrix metalloproteinase inhibitors with antitumour activity. *Bioorg Med Chem* **2003**, 11, 5461-5484.
7. Lu P, Liu J, Wang Y, Chen X, Yang Y, Ji R. Design, synthesis and evaluation of novel oxazaphosphorine prodrugs of 9-(2-phosphonomethoxyethyl)adenine (PMEA, adefovir) as potent HBV inhibitors. *Bioorg Med Chem Lett* **2009**, 19, 6918-6921.
8. Paar WD, Frankus P, Dengler HJ. The metabolism of tramadol by human liver microsomes. *Clin Investig* **1992**, 70, 708-710.
9. Choi S, Sainz Jr B, Corcoran P, Uprichard S, Jeong H. Characterization of increased drug metabolism activity in dimethyl sulfoxide (DMSO)-treated Huh7 hepatoma cells. *Xenobiotica* **2009**, 39, 205-217.

CHAPTER EIGHT

Phosphorodiamidates- phosphate prodrugs.

8.1 Phosphorodiamidate prodrugs.

As already mentioned in Chapter 2, diamidate prodrugs are a group of pronucleotides capable of entering the cell by passive cellular uptake.¹ In the phosphorodiamidate core structure, the phosphate group is masked with two amines (including amino acids). Considering the structure of phosphorodiamidates two families can be distinguished: symmetrical diamidates where the phosphate group is masked with two identical amines and asymmetrical diamidates where two distinctive amines are attached to the phosphorus atom (Figure 8.1).

Symmetrical phosphorodiamidates have two main advantages over aryl phosphoramidates. The first one relates to the phosphorus stereogenic centre. Since two identical masking groups are used to mask the phosphate, it is no longer chiral and issues associated with diastereoisomer formation during the synthesis do not exist.

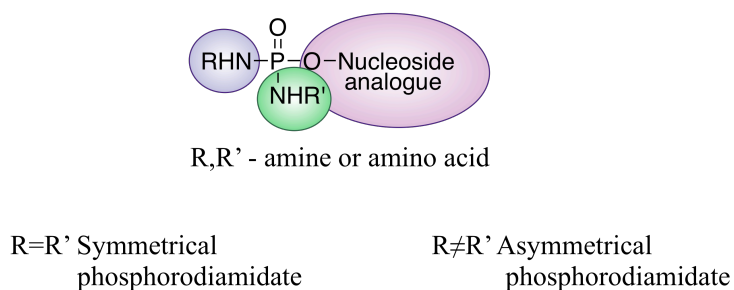
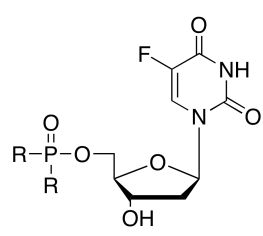


Figure 8.1. General structure of phosphorodiamidate. R, R'=amine or amino acid.

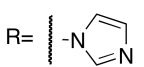
The second advantage relates to the toxicity of synthesised prodrugs. When amino acid esters are used as phosphate masking moieties, the by-products of the enzymatic activation consist of non-toxic amino acids (at released concentrations).

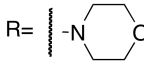
The first reported family of nucleoside analogue phosphorodiamidates consisted of a series of 5'-phosphorodiamidates of FUDR. Different amines were used as masking units, and compounds were evaluated against murine leukaemia L5178Y cell line.² A positive correlation between compound activity and the rate of hydrolysis of diamidates was found.

Phosphorodiamidate [FUdR-5'-P(NH₂)₂] was found to be the most active against L5178Y cell line and the least stable in the hydrolytic assay. The diamidate bearing two morpholine units was found to be the least active and the most stable at the same time (Figure 8.2).²



R= NH₂

R= 

R= 



| R | % of inhibition at 10 nM | T _{1/2} hydrolysis to FdU-5'P at 100 °C [h] |
|---|--------------------------|--|
| OH | 67% | - |
| NH ₂ | 45% | 3.9 |
|  | 16% | 12 |
|  | 11% | 100 |

Figure 8.2. Structures of FUdR diamidates. Inhibition of L5178 cell growth *in vitro* and rate of hydrolysis to FdU-5'P.

Although *in vitro* studies proved the concept of intracellular monophosphate delivery, this class of phosphorodiamidate prodrugs was not greatly developed.

Although diamides have existed in the literature for almost 20 years, they have only recently emerged as an important class of prodrugs. McGuigan *et al.* reported application of the phosphorodiamidate approach to AZT and FdT.^{1,3} In the case of AZT 5'-diamidates, the amino acid derivatives were particularly active comparing to the primary and secondary amine derivatives. However, the potency of these compounds was lower than the parent nucleoside.³ Interestingly, clear correlation between the toxicity of masking units was observed. The amino acid derivatives were found to be non-toxic while secondary amine analogues possess marked toxicity.³

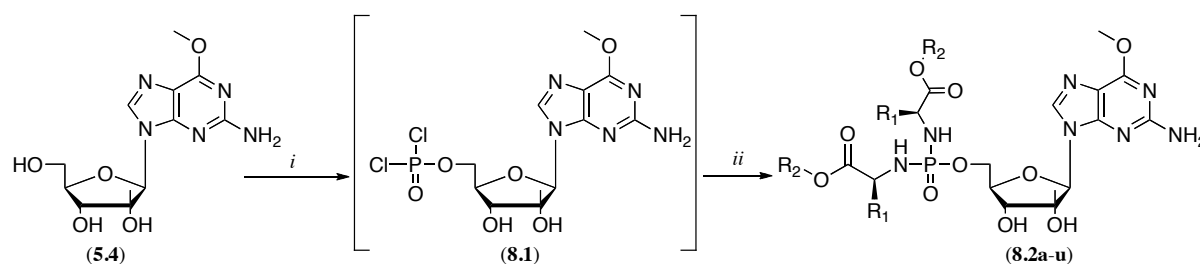
The initial studies of the phosphorodiamidate technology applied to the PMEA analogue 9-[2-(phosphonomethoxy)ethoxy]adenine were unsuccessful. The prodrug was prepared as a glycine methyl ester and failed to afford any detectable blood levels of the active drug following oral administration to mice.⁴ Recently PMEA diamidates were found to exhibit inhibitory activity against orthopoxvirus replication *in vitro*. These results indicate effective cellular permeation and intracellular release of the corresponding PMEA 5'-phosphonate.⁵

We decided to apply a phosphorodiamidate approach to the 2-amino-6-methoxy-9-(2'-C-methyl-β-D-ribofuranosyl) purine in order to explore its effect on anti-HCV activity.

8.2 Synthesis of symmetrical amino acid phosphorodiamidates of 6-*O*-methyl-2'-*C*- β -methylguanosine.

There are several reported synthetic pathways of phosphorodiamidate synthesis.¹ In our study we decided to adapt the Yoshikawa⁶ procedure to obtain 5'-phosphorylated nucleoside analogue using unprotected nucleoside 6-*O*-methyl-2'-*C*- β -methylguanosine.

In one pot reaction, free nucleoside analogue **5.4** (Scheme 8.1) was reacted in anhydrous conditions with POCl₃ and Et₃N in dry THF, to give 5'-phosphorylated intermediate **8.1** (Scheme 8.1). The disappearance of the POCl₃ ³¹P signal and formation of the new peak at ~8.00 ppm was followed by the ³¹P NMR. The phosphorylation step was found to be critical for the formation of phosphorodiamidates. It was found that the intermediate **8.1** forms within the first 30 min of the reaction time. When reaction time was prolonged, degradation/hydrolysis of **8.1** was observed. Due to the relative instability of **8.1**, the compound was not isolated. The amino acid ester was added to the reaction mixture, followed by the addition of dry dichloromethane and triethylamine. The progress of the reaction was monitored by the phosphorus NMR. Appearance of the single peak at ~13-17 ppm (depending on amino acid used for the synthesis (Table 8.1)) indicated formation of the product. The overnight reaction followed by the purification on silica gel resulted in a final phosphorodiamidate ProTides **8.2a-u** (Scheme 8.1 and Table 8.1) in moderate to good yields.



Reagents and Conditions: i) POCl₃, Et₃N, dry THF, -78 °C to rt, 30 min; ii) Appropriate amino acid ester, Et₃N, DCM, -78 °C to rt, overnight.

Scheme 8.1. Synthesis of symmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine.

In some cases additional extraction with 0.1 M HCl was required in order to remove excess of amino acid esters.

As the structure activity relationship of symmetrical phosphorodiamidates was unknown, it was decided to vary amino acids and ester units (Table 8.1).

| Cpd | R ₁ | AA | R ₂ (ester) | ClogP | ³¹ P [δ ppm] MeOD | Yield %* |
|----------------|---|-------|------------------------|-------|---------------------------------|----------|
| 8.2a ** | CH ₃ | L-Ala | <i>n</i> -propyl | 2.6 | 14.00 | 7 |
| 8.2b | CH ₃ | L-Ala | <i>n</i> -butyl | 3.7 | 14.02 | 14 |
| 8.2c | CH ₃ | L-Ala | <i>n</i> -pentyl | 4.7 | 14.05 | 40 |
| 8.2d | CH ₃ | L-Ala | <i>R,S</i> -2-butyl | 3.2 | 14.05 | 17 |
| 8.2e | CH ₃ | L-Ala | 2,2-dimethylpropyl | 4.2 | 14.00 | 32 |
| 8.2f | CH ₃ | L-Ala | 3,3-dimethylbutyl | 5.2 | 14.03 | 9 |
| 8.2g ** | CH ₃ | L-Ala | cyclohexyl | 4.6 | 14.07 | 6 |
| 8.2h | CH ₃ | L-Ala | tetrahydropyranyl | -0.2 | 13.94 | 14 |
| 8.2i ** | CH ₃ | L-Ala | benzyl | 3.9 | 13.93 | 9 |
| 8.2j | CH ₃ | L-Ala | 2-indanyl | 4.4 | 13.94 | 9 |
| 8.2k | CH(CH ₃) ₂ | L-Val | 2,2-dimethylpropyl | 6.1 | 15.16 | 6 |
| 8.2l | CH(CH ₃) ₂ | L-Val | cyclohexyl | 6.4 | 15.28 | 5 |
| 8.2m | CH(CH ₃) ₂ | L-Val | benzyl | 5.8 | 15.11 | 10 |
| 8.2n | CH(CH ₃) ₂ | L-Ile | methyl | 3.4 | 14.88 | 22 |
| 8.2o | CH(CH ₃)CH ₂ CH ₃ | L-Ile | 2,2-dimethylpropyl | 7.2 | 14.93 | 29 |
| 8.2p | CH(CH ₃)CH ₂ CH ₃ | L-Ile | cyclohexyl | 7.5 | 15.04 | 19 |
| 8.2q | CH(CH ₃)CH ₂ CH ₃ | L-Ile | benzyl | 6.9 | 14.93 | 16 |
| 8.2r ** | CH ₂ CH ₂ SCH ₃ | L-Met | 2,2-dimethylpropyl | 4.5 | 14.36 | 12 |
| 8.2s | CH ₂ CH ₂ SCH ₃ | L-Met | cyclohexyl | 4.8 | 14.26 | 20 |
| 8.2t | CH ₂ CH ₂ SCH ₃ | L-Met | benzyl | 4.2 | 14.11 | 16 |
| 8.2u | - | β-Ala | benzyl | 3.9 | 17.45 | 25 |

ClogP: calculated lipophilicity, values calculated using ChemOffice Ultra 11.0; *Yields calculated over two steps. ** Synthesised by M. Aljarah.⁷

Table 8.1 Summary of symmetrical diamidates synthesised, ClogP values, ³¹P NMR shifts and synthetic yields.

Based on our experience with phosphoramidates of 6-*O*-methyl-2'-*C*-methylguanosine, we decided to investigate L-alanine amino acid first. Different ester units including linear, branched, cyclic and aromatic were explored (Table 8.1, entries **8.2a-j**). The compound **8.2e** was prepared as a diamidate analogue of **INX-189** in order to compare the efficiency of these two prodrug approaches.

Although the L-alanine amino acid proved to be strongly preferred in phosphoramidate series, it was decided to investigate other amino acids as well and evaluate their effect on antiviral potency of 6-*O*-methyl-2'-*C*-methylguanosine phosphorodiamidates. A series of L-valine (**8.2k-m**), L-isoleucine (**8.2n-q**) and L-methionine (**8.2r-t**) compounds was prepared. In order to facilitate comparison compounds were synthesised as 2,2-dimethylpropyl, cyclohexyl and benzyl esters. Additionally an example of β -alanine phosphorodiamidate **8.2u** was made with the aim of probing the activation mechanism.

The variation of different amino acids and ester moieties lead to the compounds with a ClogP values between -0.2 and 7.5 (Table 8.1). It was interesting to see, whether the same tendency like in the case of 6-*O*-methyl-2'-*C*-methylguanosine aryloxyphosphoramidates would be observed. As described in Chapter 5, the most potent compounds possess ClogP values in a range between 2 and 4. Compounds with ClogP values below 2 and above 4 had lower potency.

8.3 Biological evaluation of symmetrical diamidates of 6-*O*-methyl-2'-*C*-methylguanosine.

All novel phosphorodiamidates were evaluated by Inhibitex Inc., for their potential as anti-HCV agents in *in vitro* replicon assay. Related data are reported in Table 8.2.

| Cpd | R ₁ | AA | R ₂ (ester) | ClogP | EC ₅₀ [μ M] | CC ₅₀ [μ M] |
|----------------|-----------------|-------|------------------------|-------|-----------------------------|-----------------------------|
| 5.4 | - | - | - | -0.6 | 4.6 | >100 |
| INX-189 | CH ₃ | L-Ala | 2,2-dimethylpropyl | 3.1 | 0.01 | 7 |
| 8.2a | CH ₃ | L-Ala | <i>n</i> -propyl | 2.6 | 0.3 | >100 |
| 8.2b | CH ₃ | L-Ala | <i>n</i> -butyl | 3.7 | 0.07 | >100 |
| 8.2c | CH ₃ | L-Ala | <i>n</i> -pentyl | 4.7 | 0.03 | 65 |
| 8.2d | CH ₃ | L-Ala | <i>R,S</i> -2-butyl | 3.2 | 0.15 | >100 |
| 8.2e | CH ₃ | L-Ala | 2,2-dimethylpropyl | 4.2 | 0.06 | >100 |
| 8.2f | CH ₃ | L-Ala | 3,3-dimethylbutyl | 5.2 | 0.02 | 71 |
| 8.2g | CH | L-Ala | cyclohexyl | 4.6 | 0.06 | >100 |
| 8.2h | CH ₃ | L-Ala | tetrahydropyranyl | -0.2 | 13.3 | >100 |
| 8.2i | CH ₃ | L-Ala | benzyl | 3.9 | 0.49 | >100 |
| 8.2j | CH ₃ | L-Ala | 2-indanyl | 4.4 | 0.58 | 70 |

| Cpd | R ₁ | AA | R ₂ (ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|-------------|---|-------|------------------------|-------|-----------------------|-----------------------|
| 8.2k | CH(CH ₃) ₂ | L-Val | 2,2-dimethylpropyl | 6.1 | 0.72 | 32 |
| 8.2l | CH(CH ₃) ₂ | L-Val | cyclohexyl | 6.4 | 2.5 | 20 |
| 8.2m | CH(CH ₃) ₂ | L-Val | benzyl | 5.8 | 0.12 | 49 |
| 8.2n | CH(CH ₃)CH ₂ CH ₃ | L-Ile | methyl | 3.4 | 5.17 | >100 |
| 8.2o | CH(CH ₃)CH ₂ CH ₃ | L-Ile | 2,2-dimethylpropyl | 7.2 | 2.9 | 15 |
| 8.2p | CH(CH ₃)CH ₂ CH ₃ | L-Ile | cyclohexyl | 7.5 | 4.0 | 14 |
| 8.2q | CH(CH ₃)CH ₂ CH ₃ | L-Ile | benzyl | 6.9 | 0.4 | 24 |
| 8.2r | CH ₂ CH ₂ SCH ₃ | L-Met | 2,2-dimethylpropyl | 4.5 | 0.09 | 69 |
| 8.2s | CH ₂ CH ₂ SCH ₃ | L-Met | cyclohexyl | 4.8 | 0.6 | 51 |
| 8.2t | CH ₂ CH ₂ SCH ₃ | L-Met | benzyl | 4.2 | 0.25 | >100 |
| 8.2u | - | β-Ala | benzyl | 3.9 | 3.8 | >100 |

AA: Amino acid, EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 8.2. Summary of the anti-HCV activity of 6OMe2'CMcG phosphorodiamidates, **INX-189** and parent nucleoside **5.4**, and their calculated ClogP values.

Parent nucleoside **5.4** and **INX-189** were tested alongside.

As can be seen in Table 8.2, most of the synthesised phosphorodiamidates were significantly more potent than the parent nucleoside **5.4**, in low nanomolar range. The most active compound across the L-alanine phosphorodiamidate family was **8.2f** (3,3-dimethyl-1-butyl derivative, EC₅₀ = 0.02 μM), followed by **8.2c** (L-alanine-*O*-pentyl, EC₅₀ = 0.03 μM), **8.2e** (L-alanine-*O*-2,2-dimethylpropyl, EC₅₀ = 0.06 μM) and **8.2g** (cyclohexyl analogue, EC₅₀ = 0.06 μM). These compounds showed 75- to 200-fold enhancement in the antiviral potency comparing to the parent nucleoside **5.4**. Phosphorodiamidate **8.2f** exhibit similar potency as **INX-189**, however **8.2f** was ca. 7 times less cytotoxic than **INX-189** in replicon assay (71 μM vs. 7 μM). The diamidate analogue of **INX-189**, **8.2e** was found to be 5-fold less active in cell based assay than its aryloxyphosphoramidate analogue but at the same time significantly less cytotoxic (CC₅₀ >100 μM vs. 7 μM). Thus, the values of the therapeutic indexes for diamidates were in the range of a 3500. This profound lack of toxicity in anti-HCV assay makes diamidates very interesting compounds for further development.

The structure activity relationship of L-alanine phosphorodiamidates revealed that *n*-alkyl ester derivatives were more potent with increasing chain length, thus *n*-pentyl **8.2c** ($EC_{50} = 0.03 \mu\text{M}$) > *n*-butyl **8.2b** ($EC_{50} = 0.07 \mu\text{M}$) > *n*-propyl **8.2a** ($EC_{50} = 0.3 \mu\text{M}$). These data clearly correlate with calculated lipophilicity for compounds **8.2a-c** (Figure 8.3), showing that the *n*-pentyl derivative, which was the most lipophilic, was also the most active.

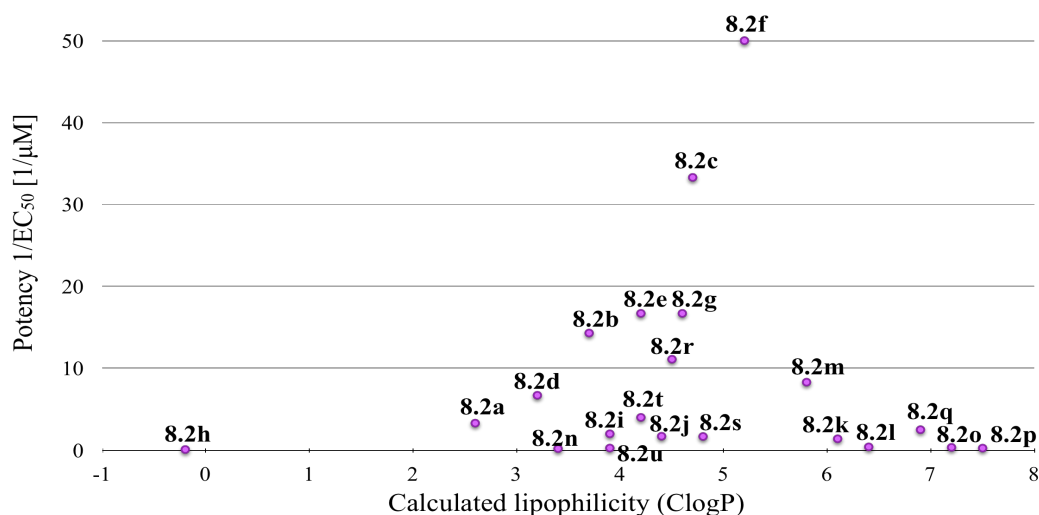


Figure 8.3. Potency ($1/EC_{50}$) and calculated lipophilicity (ClogP) data plot of synthesised phosphoramidates **8.2a-j**.

Not much difference in anti-HCV activity between linear and β or γ branched esters was found. Hence β -branched 2,2-dimethylpropyl analogue **8.2e** possesses similar activity as *n*-butyl **8.2b** analogue ($EC_{50} = 0.06 \mu\text{M}$ vs. $0.07 \mu\text{M}$) and activity of γ -branched 3,3-dimethyl-1-butyl **8.2f** compound was comparable to *n*-pentyl **8.2c** ($EC_{50} = 0.02 \mu\text{M}$ vs. $0.03 \mu\text{M}$). Similarly, like in the case of linear esters, longer branched esters possessed increased activity comparing to the shorter derivatives, which also correlates with ClogP values for these compounds. Sec-butyl-L-alanine phosphoramidate **8.2d** was found to be ca. 7-fold less active than **8.2f** ($EC_{50} = 0.15 \mu\text{M}$ vs. $0.02 \mu\text{M}$).

In case of cyclic esters, the cyclohexyl compound **8.2g** ($EC_{50} = 0.06 \mu\text{M}$) was as potent as **8.2b** or **8.2e**. Tetrahydropyranyl analogue **8.2h** was found to be the least active across the whole synthesised family of phosphorodiamidates. This particular compound was found to be less active than the parent nucleoside **5.4** ($EC_{50} = 13.3 \mu\text{M}$ vs. $4.6 \mu\text{M}$, Table 8.2). The poor activity of **8.2h** can be explained by the low ClogP value, suggesting that

compound is not getting efficiently inside the cell and thus the intracellular release of the 5'-monophosphate is greatly limited.

Surprisingly L-alanine benzyl ester derivative **8.2i** ($EC_{50} = 0.49 \mu\text{M}$) was found to be less active than most of the *n*-alkyl ester analogues. This is in contrast with aryloxyphosphoramidate data reported in Chapters 4 and 5, where benzyl derivatives were amongst the most potent compounds. These findings cannot be explained by the lipophilicity of the compound, as it is similar to *n*-butyl analogue **8.2b** ($\text{ClogP} = 3.9$ vs 3.7). The indanyl analogue **8.2j** exhibited similar activity as **8.2i** ($EC_{50} = 0.58 \mu\text{M}$ vs. $EC_{50} = 0.49 \mu\text{M}$).

Structure activity relationships of 6-*O*-methyl-2'-*C*-methylguanosine diamidates bearing different amino acids are presented in Table 8.2, entries **8.2k-u**, and Figure 8.4. More bulky amino acids were considered as more stable towards enzymatic degradation. All compounds in general were less active than L-alanine analogues. Across L-valine series, the most potent compound was bearing benzyl ester and was found to be ca. 4-times more potent than L-alanine analogue ($EC_{50} = 0.12 \mu\text{M}$ vs. $EC_{50} = 0.49 \mu\text{M}$). These data are in contrast with phosphoramidate ProTide results, where L-alanine benzyl ester **4.13i** was ca. 10-fold more potent than L-valine analogue **4.13m** ($EC_{50} = 0.06 \mu\text{M}$ vs. $EC_{50} = 0.76 \mu\text{M}$, respectively). Whereas cyclohexyl- and 2,2-dimethylpropyl-L-valine diamidates were significantly less active (Figure 8.4).

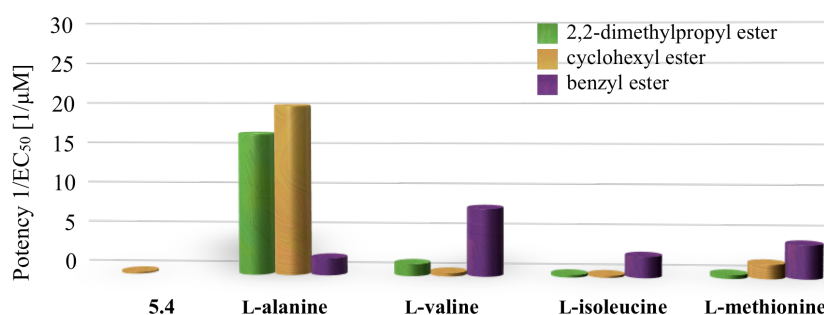


Figure 8.4. Potency ($1/EC_{50}$) data plot of various amino acid ester diamidates of 6OMe2'CMeG.

Other β -branched amino acid - L-isoleucine was found to be poorly active, with replicon EC_{50} values similar to the parent nucleoside **5.4**. The only compound that exhibited submicromolar activity in this family was benzyl derivative **8.2q** ($EC_{50} = 0.4 \mu\text{M}$), being 10-fold more potent than the parent nucleoside **5.4**. Benzyl analogue was also found to be the most active in the L-methionine series **8.2t**, $EC_{50} = 0.25 \mu\text{M}$.

The β -alanine analogue **8.2u** and parent nucleoside **5.4** were found to be equipotent. These data clearly confirm that position of carboxyl group in relation to the amine is very important for activity.

From the SAR studies general conclusions can be made, namely that the small amino acids e.g. L-alanine are strongly preferred over longer or branched amino acids. For amino acids different than L-alanine, benzyl ester derivatives were found to be the most potent (Figure 8.4).

Considering the ClogP of synthesised compounds, some consistencies have been observed. Compounds that exhibit ClogP values below 3.4 and greater than 5.5, were found to be less active than compounds with the calculated lipophilicity in the range of 3.5-5.5 (Figure 8.3). It is possible that compounds with the high logP values can be too lipophilic to cross the cellular membrane and can get trapped in the lipid bilayer, and as a consequence low levels of intracellular 5'-triphosphate are generated. This could explain lower antiviral potency for L-isoleucine compounds (**8.2o-q**, Figure 8.3). Solubility may also be an issue in biological assays.

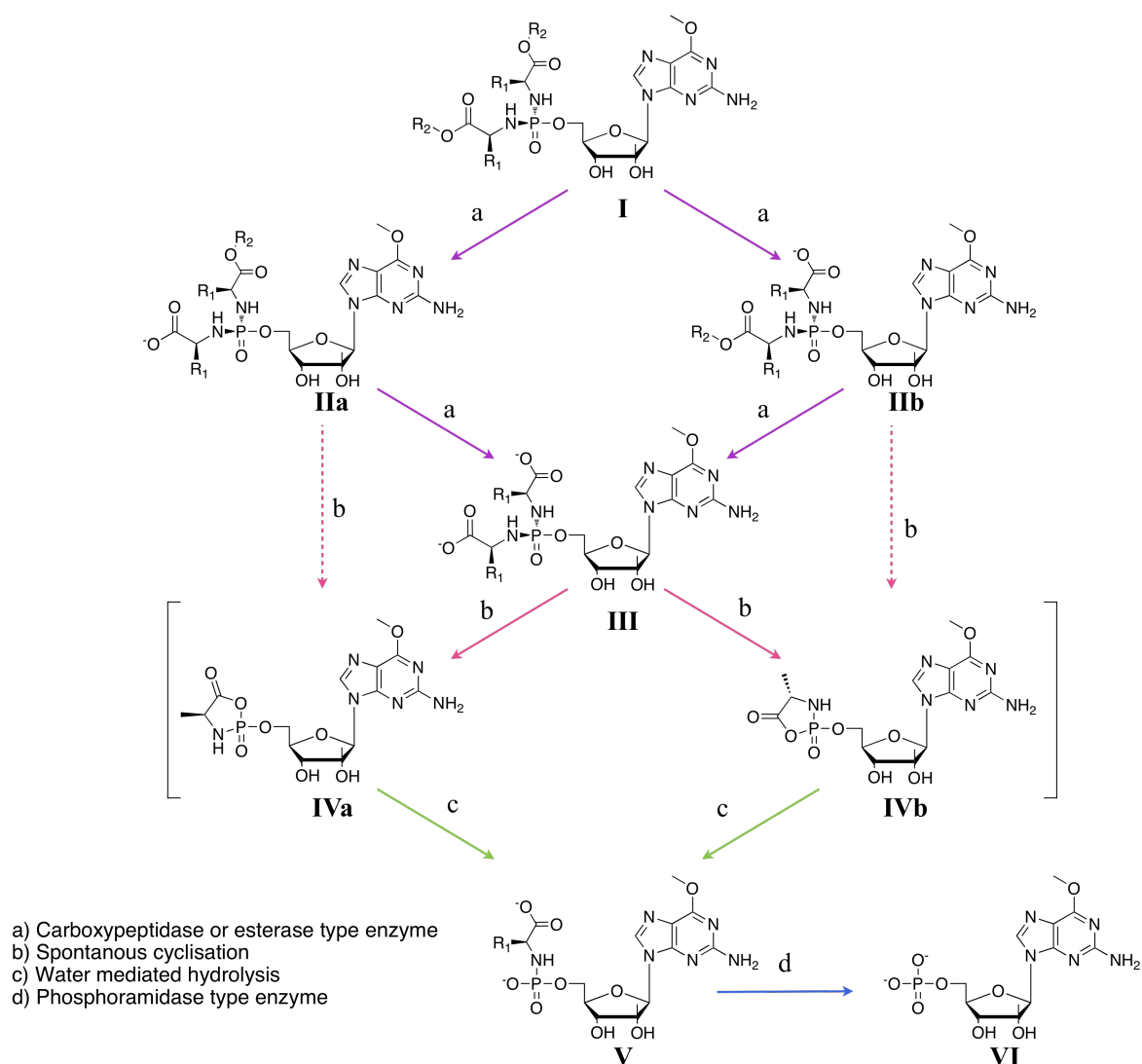
In general, symmetrical diamidates tested in the replicon assay were found to be less toxic than analogous phosphoramidates of 6-*O*-methyl-2'-*C*-methylguanosine (refer to Chapter 5), with CC₅₀ values greater than 100 μ M. However some cellular toxicity for compounds bearing different amino acids than L-alanine was observed. For example cyclohexyl-L-isoleucine **8.2p** and 2,2-dimethylpropyl-L-isoleucine **8.2o** compounds were found to be toxic at 14 μ M and 15 μ M concentrations, respectively. Considering the anti-HCV activity of these compounds, their respective selectivity indexes would be ca. 4 for **8.2p** and 5 for **8.2o**, and would make them one of the most cytotoxic across diamidate series.

8.4 Enzymatic studies of symmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine.

To probe the mechanism of activation of symmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine several enzymatic assays were performed.

8.4.1 Putative mechanism of activation of symmetrical diamidates.

The proposed mechanism of activation of phosphorodiamidate prodrugs would involve ester cleavage of one of the masking units (IIa, IIb Scheme 8.2) followed by the intramolecular attack of the amino acid carboxylate anion onto the phosphorus with subsequent cyclisation (IVa, IVb) and elimination of the second amino acid (V, Scheme 8.2). It is not clear if both of the esters are hydrolysed at the same time (III) or only one of them. Hydrolysis of the unstable five-membered ring would result in formation of the final aminoacyl intermediate (V). The final conversion and release of the monophosphate is thought to relay on Hint enzyme. The 5'-monophosphate (VI) could then undergo further phosphorylation to the active 5'-triphosphate form.



Scheme 8.2. Putative mechanism of activation of 6OMe2'CMcG phosphorodiamidates.

8.4.2 Carboxypeptidase Y studies of symmetrical diamidates.

As already mentioned, the initial step of the activation of diamidates involves an enzyme-mediated hydrolysis of the amino acid ester moiety. Thus, to probe the activation pathway, an enzymatic assays in the presence of carboxypeptidase Y, an enzyme capable of *in vitro* ester cleavage, were performed.

The first assay was conducted on L-alanine benzyl ester derivative **8.2i**, according to the procedure described in Chapter 4, Section 4.6.

In the blank ^{31}P spectrum, a single peak at $\delta_{\text{P}} = 14.08$ ppm corresponds to the parent phosphorodiamidate. After addition of the enzyme, rapid hydrolysis of the ester units was observed, with complete disappearance of the starting material within 30 min (Figure 8.5).

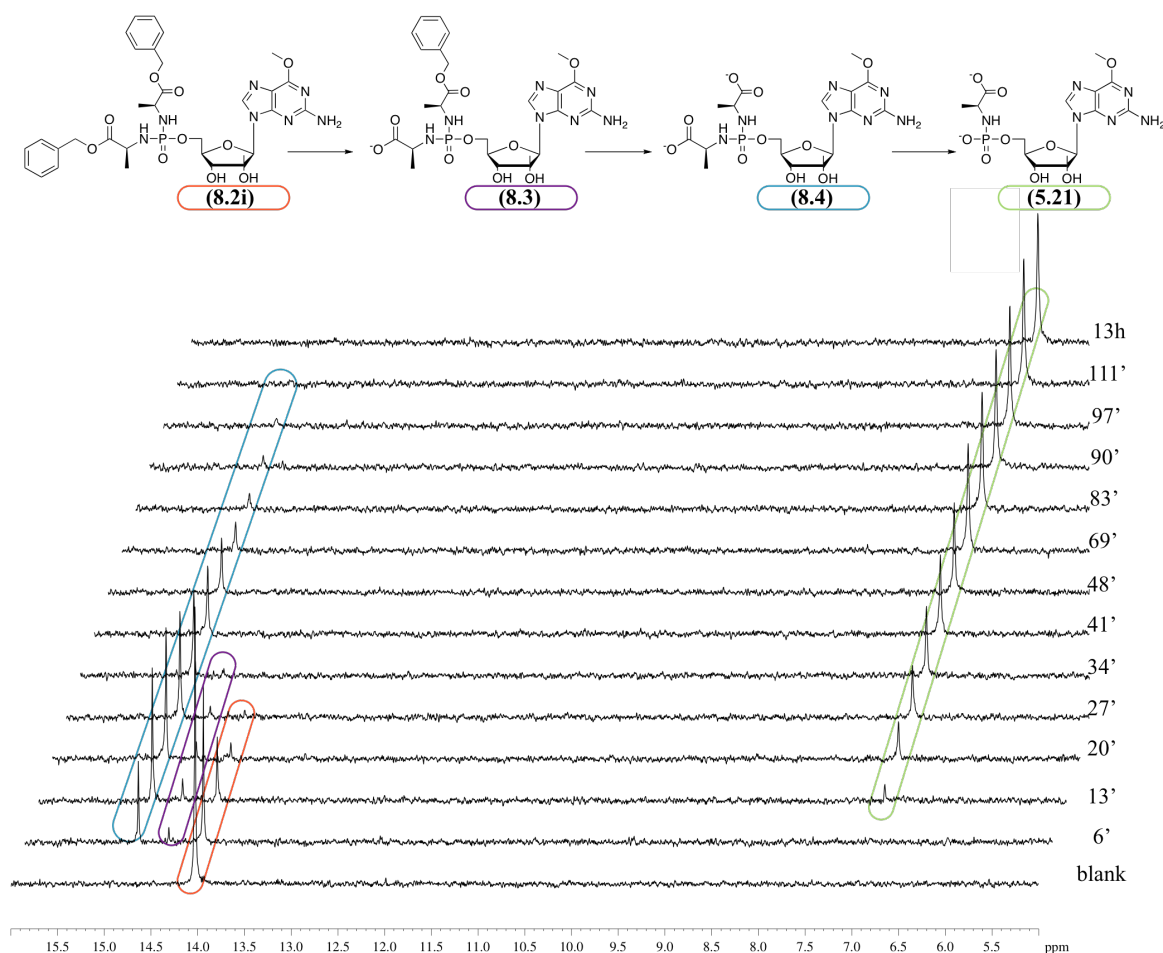


Figure 8.5. Carboxypeptidase Y mediated hydrolysis of **8.2i**, followed by ^{31}P NMR, with proposed structures.

The chemical structures depicted in Figure 8.5, represent one of the possible routes leading to the formation of aminoacyl derivatives **5.21**. The intermediate with ^{31}P chemical

shift of 14.46 ppm, could correspond to the structure where only one of the ester moieties is hydrolysed **8.3**, while signal at $\delta_P = 14.78$ ppm to the compound with both ester moieties being cleaved **8.4** (Figure 8.5). The compound represented by the structure **8.3** is chiral and therefore signals of pro-Rp and pro-Sp isomer would have to overlap to be represented by a single peak in the ^{31}P NMR. The other possibility is that each of the peaks: $\delta_P = 14.46$ ppm and $\delta_P = 14.78$ ppm represents a distinctive structure, where one of the esters is cleaved (**8.3a** and **8.3b**, respectively Figure 8.6). In this case the cleavage of both esters does not occur. Current data do not discriminate between these possibilities.

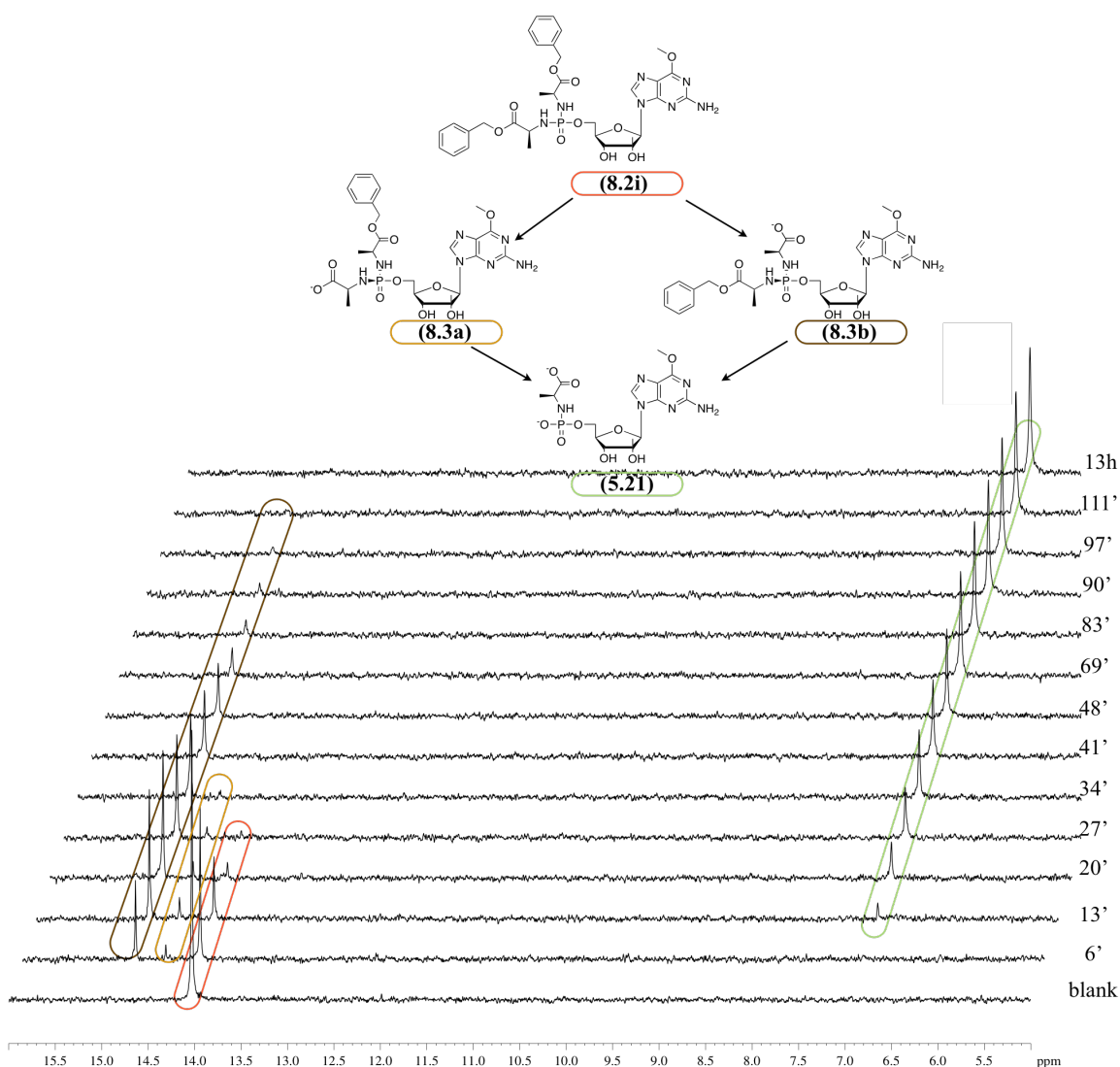


Figure 8.6. Carboxypeptidase Y mediated hydrolysis of **8.2i**, followed by ^{31}P NMR, with proposed structures.

The metabolite peak at $\delta_P = 14.46$ ppm builds up for approximately 35 min and then diminishes. The metabolite at $\delta_P = 14.78$ ppm starts to form within 6 min after carboxypeptidase Y addition and remains present for ca. 2 h.

In both cases, the cyclisation of **8.3**, **8.3a** and **8.3b** with subsequent elimination of one of the amino acids results in the formation of **5.21** (Figure 8.5 and 8.6). The intermediate **5.21** starts to form within 13 min of incubation. The presence of the peak at 6.94 ppm is consistent with the chemical shift of the identical amino acyl intermediate released during enzymatic activation of aryloxyphosphoramidates of 6-*O*-methyl-2'-*C*-methylguanosine (Chapter 5, Section 5.5.2). The half-life of **8.2i** was found to be less than 13 min.

In the case of the 2,2-dimethylpropyl-L-alanine diamidate **8.2e**, subtle differences in the processing pattern can be seen (Figure 8.7)

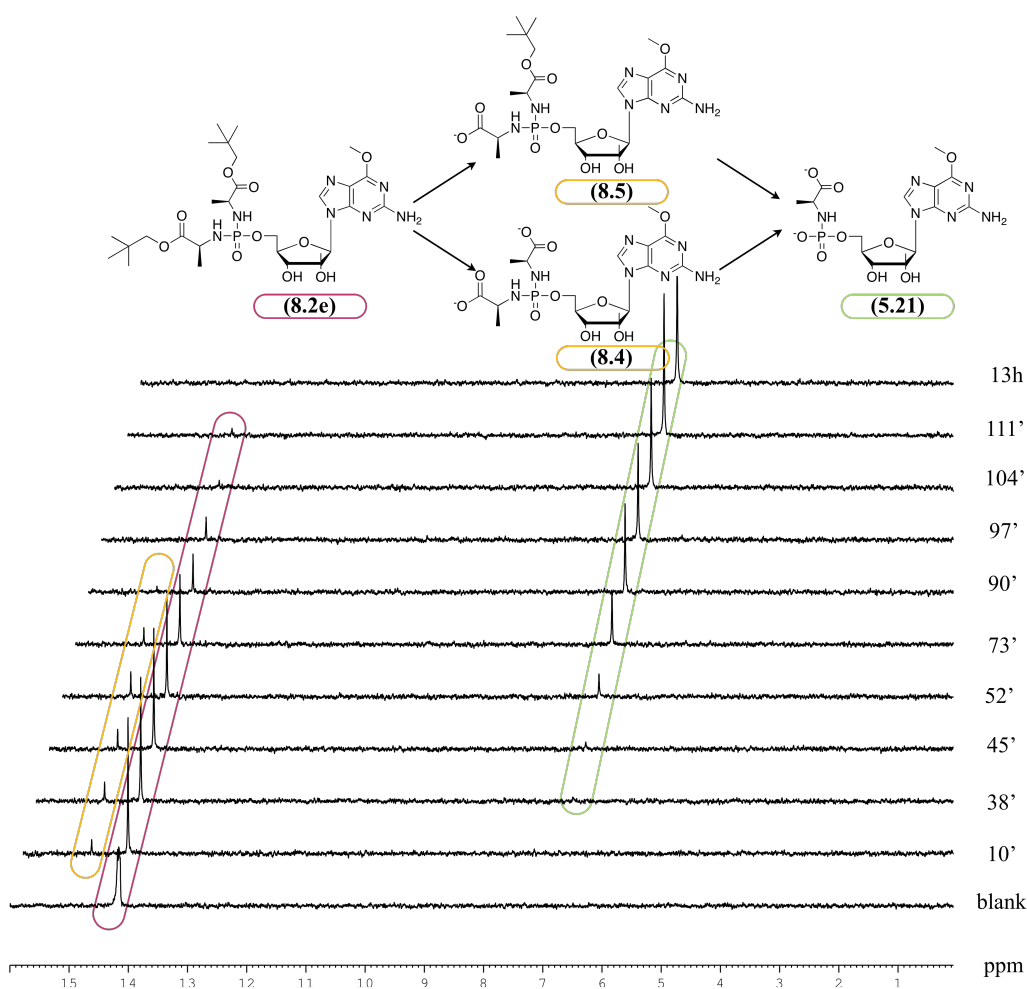


Figure 8.7. Carboxypeptidase Y mediated hydrolysis of **8.2e**, followed by ^{31}P NMR, with proposed structures.

After 10 min of incubation of parent diamidate ($\delta_P = 14.24$ ppm) in the presence of carboxypeptidase Y, more downfield peak at $\delta_P = 14.91$ ppm has been observed. This particular peak can correspond to the structure of metabolite **8.4** or **8.5**. The peak at 14.91 ppm builds up for approximately 40 min and after that time diminishes with simultaneous formation of **5.21** ($\delta_P = 6.95$ ppm). The half-life of **8.2e** was found to be ca. 50 min and within 2 hours the parent diamidate prodrug has been processed to the free diacid intermediate.

It has to be noted that in the case of aryloxyphosphoramidates the carboxypeptidase Y assays clearly correlated with replicon EC_{50} values. However in the case of diamidates **8.2e** and **8.2i**, where **8.2e** is ca. 10-fold more potent than **8.2i**, the overall rate of carboxypeptidase processing for both prodrugs was found to be very similar and therefore do not correlate directly with replicon potency.

8.4.3 Huh7 cell lysate studies of symmetrical diamidates.

In order to investigate the process of 5'-monophosphate release, compound **8.2b** was incubated in the presence of Huh7 cell lysate.

The compound **8.2b** was dissolved in D₂O and Trizma buffer (pH 7.6) and blank ³¹P spectrum was recorded at 37 °C. Then Huh7 cell lysate was added and spectra were recorded in 1 h intervals for 14 h at 37 °C. Corresponding data are reported in Figure 8.8.

As can be seen in Figure 8.8, after 1 h of incubation, parent diamidate **8.2b** ($\delta_P = 14.67$ ppm) is metabolized to the intermediate represented by the small downfield peak $\delta_P = 15.11$ ppm. This particular species can correspond to one of the structures, discussed in the earlier paragraph, where ester(s) unit is/are hydrolysed. At the same time, formation of the new peak at 7.11 ppm has been observed. This intermediate corresponds to the structure of aminoacyl **5.21**. After 2 h the ³¹P signal of **5.21** is no longer observed, while a new peak emerged at 1.21 ppm. Based on the ³¹P shift the new peak represents 6-O-methyl-2'-C-methylguanosine 5'-monophosphate **7.8**. This was determined by using synthesised compound **7.8** as an analytical reference standard (data not shown) under the same experimental conditions. This is the first direct observation of nucleoside monophosphate production by a diamidate. During the incubation period, the presence of a new, unknown

species at ~2.7 ppm has been observed. This compound forms within 1 h after cell lysate addition and slowly diminishes over 14 h.

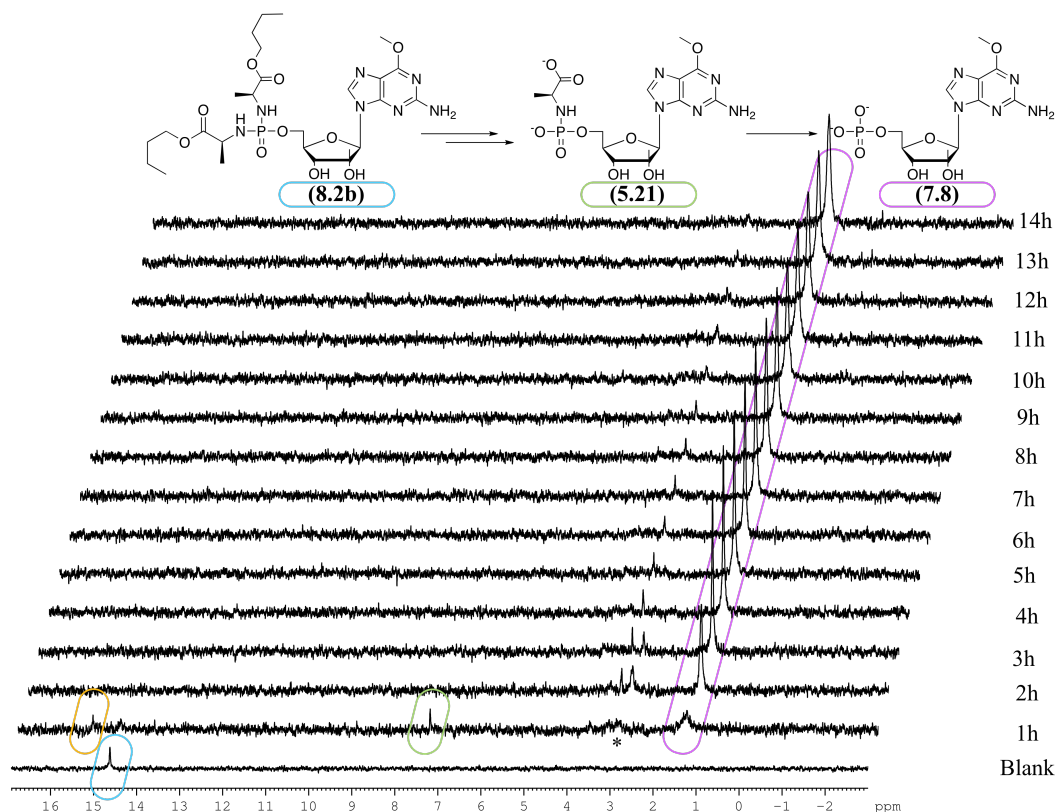


Figure 8.8. Huh7 cell lysate mediated hydrolysis of **8.2b**, followed by ^{31}P NMR, with proposed structures. * Unknown species.

Reported data may indicate ease of the monophosphate release from the phosphorodiamidate prodrug. Given that the release of the 5'-monophosphate is considered to be essential for the antiviral activity (via the 5'-triphosphate formation), these data are consistent with replicon EC_{50} values (Table 8.2).

This is the first time where ^{31}P NMR of Huh7 cell lysate was used as an *ex vivo* model of phosphorodiamidate activation.

8.5 Synthesis of asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*- β -methylguanosine.

During carboxypeptidase Y studies reported in Section 8.4.2, it has been proved that the metabolic intermediates **8.3**, **8.4** or **8.5** can successfully eliminate an amino acid moiety in order to give aminoacyl intermediate **5.21**. We therefore decide to synthesised a family of

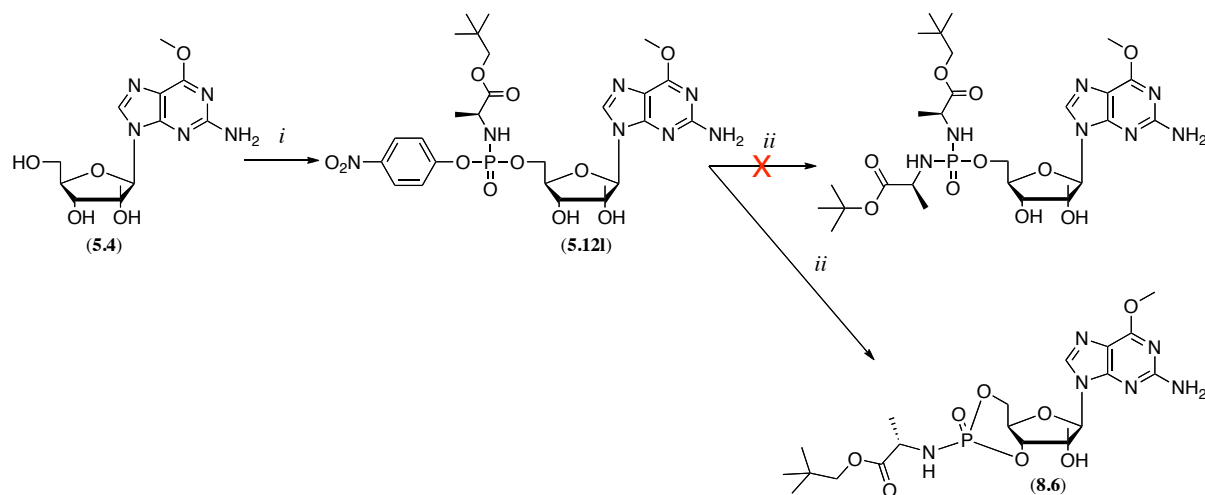
mixed diamidates consisted of amino acid and either simple primary or secondary amine, to investigate whether the latter ones can serve as a leaving groups. Additionally diamidates bearing two different amino acids were considered.

This concept was at variance with the biggest advantages of symmetrical diamidates. Introduction of the second amino acid will restore chirality on the phosphorus centre and further introduction of an amine may have an impact on toxicity of these compounds. However, from the SAR point of view, we thought that this is necessary to understand fully the nature of new phosphorodiamidate motif.

To facilitate comparison, one of the two amines was maintained either as L-alanine-*O*-2,2-dimethylpropyl ester or L-alanine-*O*-benzyl ester.

8.5.1 Synthesis of asymmetrical phosphorodiamidates via *p*-NO₂-phenyl route.

The first synthetic route consisted of coupling reaction between parent nucleoside 6-*O*-methyl-2'-*C*-methylguanosine **5.4** and *p*-NO₂-phenyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **3.3o** (Scheme 8.3).



Reagents and Conditions: i) **3.3o**, *t*BuMgCl, dry THF, rt, overnight; ii) L-alanine *tert*-butyl ester tosylate salt, DIPEA, -78 °C to rt, overnight.

Scheme 8.3. Attempted synthesis of asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine via *p*-NO₂-phenyl route.

The *p*-NO₂-phenyl group is considered as a very good leaving group and therefore it was suggested that the introduction of the second amino acid ester would proceed via nucleophilic attack of the amino group of amino acid on the phosphorus atom with

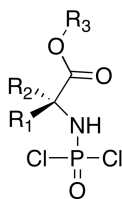
subsequent release of *p*-NO₂-phenol. Reaction was carried out in the presence of L-alanine *tert*-butyl ester tosylate salt, diisopropylethylamine (DIPEA) as a base in dry THF at ambient temperature, overnight. After 16 h no product formation was observed. Increase of the reaction time did not result in product formation. After three days of stirring reaction was warmed up to 50 °C and stirring was continued for 24 hours. After that time ³¹P NMR indicate presence of several new potential compounds. Crude mixture was purified on silica gel using CHCl₃/MeOH (0 to 7% gradient). None of the isolated fraction contain desired diamide product. However, reaction provided 3',5'-cyclic phosphoramidate **8.6** in a low yield (Scheme 8.3).

Although the *p*-NO₂-phenyl route did not result in a formation of desired asymmetrical-mixed diamidates, it demonstrates a new and alternative synthetic approach towards 3',5'-cyclic phosphoramidates.

8.5.2 Synthesis of asymmetrical phosphorodiamidates via phosphorodichloridate route.

It was decided to investigate other synthetic pathways in order to obtain mixed diamidates. Firstly, the approach via an amino acid chloridate intermediate was considered.

The L-alanine 2,2-dimethylpropyl/benzyl ester phosphorodichloridates used for the synthesis were prepared using the following procedure: Anhydrous Et₃N was added dropwise at -78 °C to a solution of the appropriate amino acid ester salt and POCl₃ in dry DCM. After 1 h at -78 °C, the reaction mixture was allowed to warm up to ambient temperature and formation of the product was followed by the ³¹P NMR. After reaction completion, solvents were removed under vacuum and the crude mixture was purified on silica gel using hexane/ethyl acetate (7:3) as an eluent. Pure compounds **8.7a-b** were obtained as yellowish oils in high yields (Table 8.3).

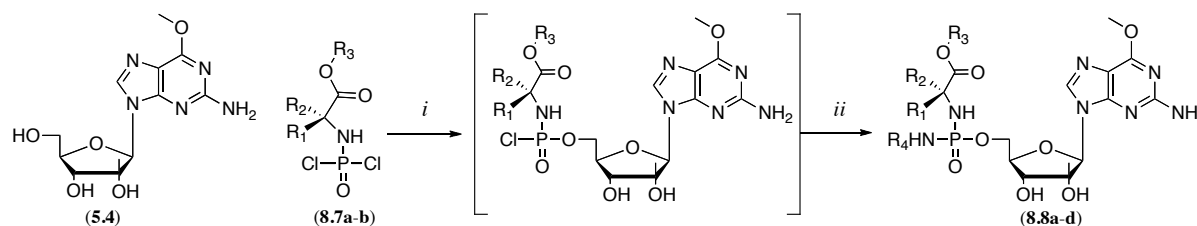


| Cpd | R ₁ | R ₂ | AA | R ₃ (ester) | ³¹ P [δ ppm] CDCl ₃ | Yield% |
|-------------|-----------------|----------------|-------|------------------------|--|--------|
| 8.7a | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 12.83 | 83 |
| 8.7b | CH ₃ | H | L-Ala | benzyl | 12.77 | 80 |

AA: Amino acid.

Table 8.3. Summary of synthesised L-alanine dichloridates **8.7a-b**.

The 6-*O*-methyl-2'-*C*-methylguanosine nucleoside **5.4** was coupled with the appropriate amino acid ester dichloridate **8.7a-b** under standard *t*BuMgCl coupling conditions. After 16 hours second amino acid was added to the reaction mixture, followed by the addition of triethylamine at -78 °C. Although the first step of reaction did work (presence of the corresponding ³¹P signals ³¹P NMR), the introduction of second amino acid ester was not successful and starting material was recovered. Therefore it was decided to use simple amines (butylamine, pentylamine, cyclopropylamine) in the second step, to investigate whether the reaction can take place. After purification of crude mixture on silica gel using CHCl₃/MeOH (0 to 5% gradient) as an eluent, asymmetrical diamidates **8.8a-d** were obtained (Scheme 8.4 and Table 8.4) as off white solids.



Reagents and Conditions: i) *t*BuMgCl, dry THF, rt, overnight; ii) appropriate amine, Et₃N, -78 °C to rt, overnight.

Scheme 8.4. Synthesis of asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine via amino acid ester phosphorodichloridate route.

| Cpd | R ₁ | R ₂ | AA | R ₃ (ester) | R ₄ (amine) | ClogP | ³¹ P [δ ppm] MeOD | Yield %* |
|-------------|-----------------|----------------|-------|------------------------|------------------------|-------|---------------------------------|----------|
| 8.8a | CH ₃ | H | L-Ala | benzyl | butylamine | 3.1 | 16.21, 16.04 | 11 |
| 8.8b | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | butylamine | 3.3 | 16.25, 16.09 | 3 |
| 8.8c | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | pentylamine | 3.8 | 16.28, 16.12 | 3 |
| 8.8d | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | cyclopropylamine | 2.3 | 16.03, 15.77 | 3 |

ClogP- calculated lipophilicity, values calculated using ChemOffice Ultra 11.0; *Yields calculated over two steps.

Table 8.4 Summary of asymmetrical diamidates synthesised via phosphorodichloridate route, their ClogP values, ³¹P NMR shifts and synthetic yields.

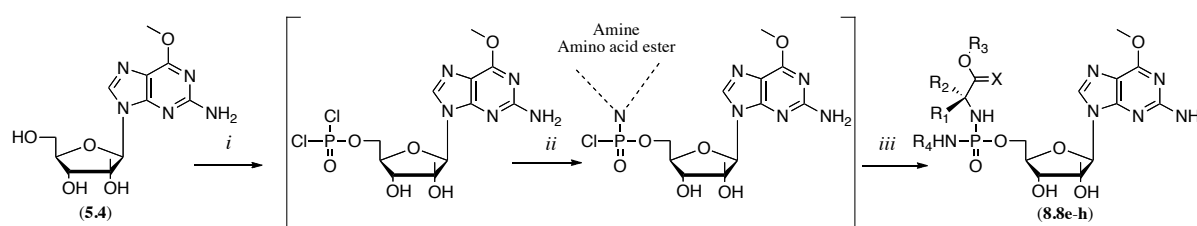
Phosphorodiamidates **8.8a-d** were isolated as ca. 1:1 mixtures of diastereoisomers at the phosphate centre.

Application of diethylamine did not result in product formation. From these findings, it was clear that this reaction pathway is suitable for the introduction of primary amines, but not secondary amines or second amino acids.

At this point, it was decided to adopt the method used for symmetrical diamidates for the requirements of mixed diamidate synthesis.

8.5.3 Synthesis of asymmetrical phosphorodiamidates via POCl₃ route.

The synthesis of asymmetrical phosphorodiamidates with chemistry methodology used for the preparation of symmetrical diamidates required stepwise introduction of one amine and one amino acid ester or two separate amino acid esters (Scheme 8.5).



Reagents and Conditions: i) POCl₃, Et₃N, dry THF, -78 °C to rt, 30 min; ii) Appropriate amine or amino acid ester, Et₃N, DCM, -78 °C to rt, 3 h; iii) Appropriate amino acid ester, Et₃N, DCM, -78 °C to rt, overnight.

Scheme 8.5. Synthesis of asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine via POCl₃ route.

The 6-*O*-methyl-2'-*C*-methylguanosine nucleoside **5.4** was allowed to react with triethylamine and phosphorus oxychloride. Formation of 5'-phosphorylated intermediate was

monitored by ^{31}P NMR. Subsequently 1 mol equivalent of amino acid ester or amine was added to the reaction mixture, followed by the addition of triethylamine. As anticipated, the product formed within 3 hours. ^{31}P NMR showed the presence of a newly formed peak at 11-12 ppm (spectra recorded in CDCl_3). The second amino acid was added to the reaction mixture, followed by the addition of triethylamine. After 16 hours, ^{31}P NMR indicated the presence of the desired product with $\delta_{\text{P}} = 12\text{-}16$ ppm (spectra recorded in MeOD). Solvents were removed under reduced pressure and crude mixture was purified on silica gel using $\text{CHCl}_3/\text{MeOH}$ (0 to 5% gradient) as an eluent.

| Cpd | R ₁ | R ₂ | AA | R ₃ (ester) | R ₄ (amine/ 2nd amino acid ester) | ClogP | ^{31}P [δ ppm] MeOD | Yield % |
|-------------|-----------------|----------------|-------|------------------------|--|-------|--------------------------------------|---------|
| 8.8e | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | L-Ala-benzyl ester | 4.1 | 13.98, 13.94 | 4 |
| 8.8f | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | diethylamine | 3.2 | 16.76, 16.68 | 3 |
| 8.8g | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | pyrrolidine | 3.8 | 14.54, 14.42 | 8 |
| 8.8h | CH ₃ | H | L-Ala | benzyl | morpholine | 2.3 | 14.65, 14.30 | 22 |

Table 8.5. Summary of asymmetrical diamidates synthesised via modified symmetrical diamidates procedure, ClogP values and ^{31}P NMR shifts.

In the case of compounds where a secondary amine was used it was important that the amine was introduced first followed by the addition of amino acid.

The purified asymmetrical phosphorodiamidates (**8.8e-h**, Table 8.5) were isolated as a roughly 1:1 mixture of diastereoisomers at the phosphorus centre, confirmed by the presence of two peaks in the ^{31}P NMR and HPLC.

8.6 Biological evaluation of asymmetrical diamidates of 6-*O*-methyl-2'-*C*-methylguanosine.

All synthesised novel asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*- β -methylguanosine were evaluated by Inhibitex Inc., against hepatitis C virus *in vitro* (Table 8.6). Additionally 3',5'-cyclic phosphoramidate **8.6**, **INX-189** and parent nucleoside **5.4** were tested alongside.

| Cpd | R ₁ | R ₂ | AA | R ₃ (ester) | R ₄ (amine/ 2nd amino acid ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|----------------|-----------------|----------------|-------|------------------------|--|-------|-----------------------|-----------------------|
| 5.4 | - | - | - | - | - | -0.6 | 4.6 | >100 |
| INX-189 | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | α-NaphO | 3.1 | 0.01 | 7 |
| 8.6 | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3',5'-cyclic | 2.0 | 0.85 | >50 |
| 8.8a | CH ₃ | H | L-Ala | benzyl | butylamine | 3.1 | 0.44 | >100 |
| 8.8b | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | butylamine | 3.3 | 0.41 | >100 |
| 8.8c | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | pentylamine | 3.8 | 0.11 | >100 |
| 8.8d | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | cyclopropylamine | 2.3 | 0.87 | >100 |
| 8.8e | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | L-Ala-benzyl ester | 4.1 | 0.15 | >100 |
| 8.8f | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | diethylamine | 3.2 | 3.7 | >100 |
| 8.8g | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | pyrrolidine | 3.8 | 0.87 | >100 |
| 8.8h | CH ₃ | H | L-Ala | benzyl | morpholine | 2.3 | 0.14 | >100 |

AA-Amino acid, EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 8.6. Summary of the anti-HCV activity of 6OMe2'CMeG asymmetrical phosphorodiamidates, 3',5'-cyclic ProTide **8.6**, **INX-189** and parent nucleoside **5.4**, and their calculated ClogP values.

As can be seen in Table 8.6, in general phosphorodiamidates bearing L-alanine and simple amines exhibited moderate to good activity in replicon based assay, with EC₅₀ values ranging from 0.1 to 0.9 μM. Compounds were found to be 5- to 40-fold more potent than the parent nucleoside **5.4**. However, compounds were found to be less active than the symmetrical L-alanine-*O*-2,2-dimethylpropyl phosphorodiamidate **8.2e** and ca. 10-100 times less potent than **INX-189**.

The most active compounds across the asymmetrical diamidates family were **8.8c** bearing primary pentylamine as a second masking group, **8.8h** L-Ala-*O*-benzyl ester/morpholine and **8.8e** mixed amino acid L-Ala-*O*-2,2-dimethylpropyl/L-Ala-*O*-benzyl diamidate, with EC₅₀ ~ 0.1 μM.

Cyclic phosphoramidate **8.6** exhibits moderate activity in *in vitro* assay (EC₅₀ = 0.85 μM), being ca. 8-fold less potent than the clinical candidate **INX-189**. Although the compound was approximately 5-times more potent than the parent nucleoside **5.4**, it was decided that further development of this particular family of cyclic phosphoramidates will not be carried out.

The least potent compound was **8.8f**, a phosphorodiamidate bearing L-Ala-*O*-2,2-dimethylpropyl ester and diethylamine as a phosphate masking units. The activity of this

particular compound was similar to the one of the parent nucleoside **5.4** ($EC_{50} = 3.7 \mu\text{M}$ vs. $EC_{50} = 4.6 \mu\text{M}$, respectively), but almost 400-fold lower than of **INX-189** (Table 8.6). Calculated ClogP of **8.8m** is identical as the one of **INX-189** and therefore cannot explain the lack of activity. In this case, decrease in replicon potency may be related to the ability of diethylamine to act as a leaving group.

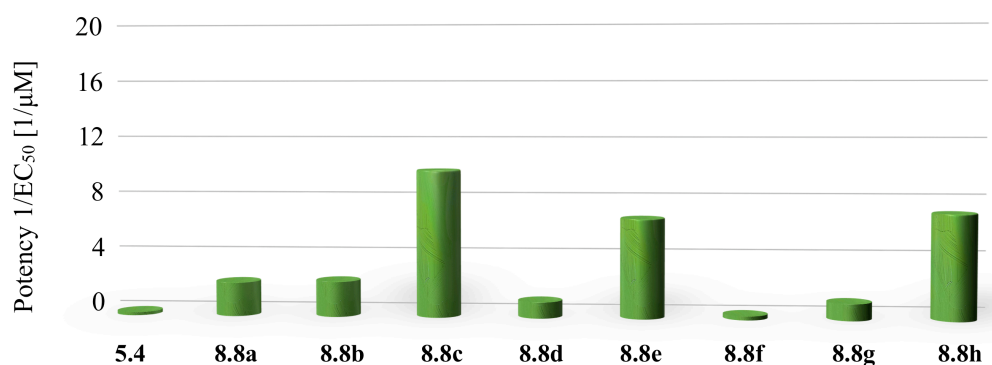


Figure 8.9. Potency ($1/EC_{50}$) data plot of diamidates bearing various simple amines.

Comparing amines between each other, it can be clearly seen (Figure 8.9) that the best choice was pentylamine **8.8c** followed by the morpholine **8.8e**, followed by the butylamine **8.8a** and **8.8b**. In general compounds bearing secondary amines were least potent than those with primary amines.

All synthesised asymmetrical diamidates exhibited no cellular toxicity up to $100 \mu\text{M}$ concentration.

In general no clear correlation between activity and calculated lipophilicity was found (Table 8.6). It appears that in the case of mixed simple amine diamidates the main feature accountable for compounds activity was leaving group ability of introduced amines. To probe these assumptions, carboxypeptidase Y-mediated ester cleavage assays were conducted.

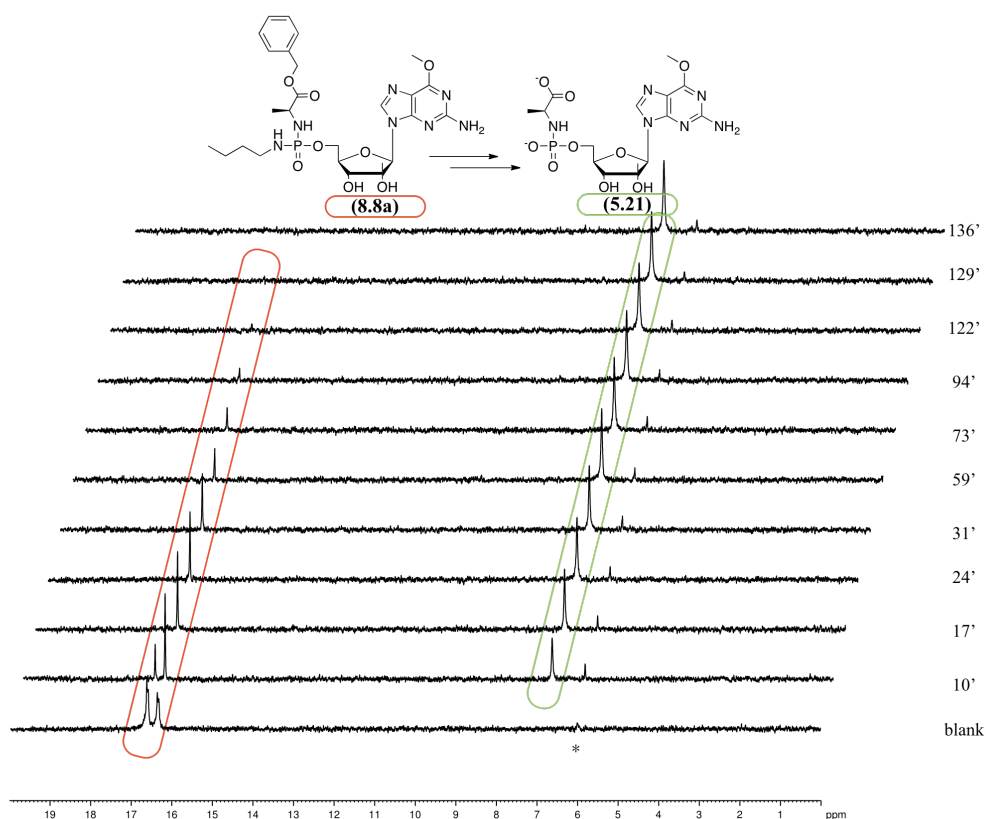
8.7 Enzymatic studies of asymmetrical phosphorodiamidates of 6-*O*-methyl-2'-*C*-methylguanosine.

The first experiment was carried out on L-alanine -*O*-benzyl/*n*-butylamine diamidate **8.8a** and corresponding data are presented in Figure 8.9.

Due to the fact that phosphorodiamidate **8.8a** is chiral at the phosphorus centre, two peaks $\delta_P = 16.75$ and 16.59 ppm are seen in the blank spectrum (Figure 8.10). Both peaks disappear within 120 min with simultaneous appearance of the new species with chemical shift of 6.94 ppm. It can be noticed that the diastereoisomer, which is represented by the more downfield ^{31}P NMR signal ($\delta_P = 16.75$) is processed faster.

The peak corresponding to the aminoacyl intermediate **5.21**, started to form within the first 10 min of incubation with carboxypeptidase Y, and builds up during the course of the experiment.

During the experiment, no signals corresponding to the species with cleaved ester moiety were observed, what could suggest that the ester cleavage followed by the spontaneous cyclisation with subsequent release of butylamine is very rapid. The half-life for **8.8a** was found to be ca. 30 min.



* Phosphate impurity in Trizma buffer; present in blank and amines unchanged throughout.

Figure 8.10. Carboxypeptidase Y mediated hydrolysis of **8.8a**, followed by ^{31}P NMR, with proposed structures.

A second experiment was performed on phosphorodiamidate **8.8g** (Figure 8.11). In the blank experiment, two peaks $\delta_P = 15.54$ and 15.42 ppm were detected. After enzyme addition a new peak at 6.94 ppm started to form within ca. 1 h and grows in magnitude over 14 h. This intermediate **5.21** is common in all phosphoramidate and phosphorodiamidate activation pathways. Interestingly, in this particular case, the enzyme cleaved only one of the two diastereoisomers of **8.8g** with rapid amine loss following the ester cleavage. The second isomer remained for over 14 hours (Figure 8.10). The half life of **8.8g** was estimated as ca. 17 hours.

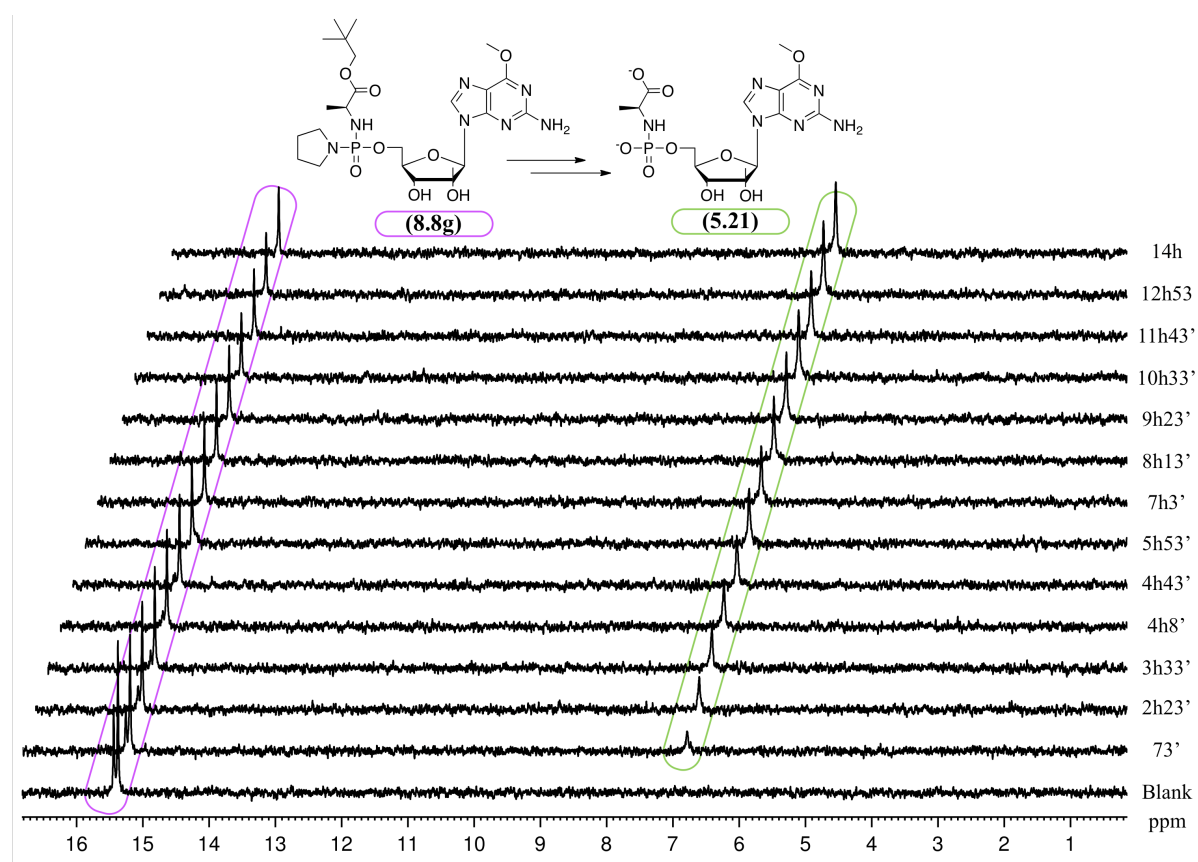


Figure 8.10. Carboxypeptidase Y mediated hydrolysis of **8.8g**, followed by ^{31}P NMR, with proposed structures.

In summary, it has been shown that a primary amine or secondary amine can also be eliminated and act as a leaving group to produce key aminoacyl intermediate **5.21**. The data obtained from the carboxypeptidase Y assays performed on **8.8a** and **8.8g** support the notion that only one ester cleavage is necessary to initiate the activation process.

8.8 Molecular modelling studies.

To understand the difference in the processing rate of the two isomers of **8.8g**, docking studies were carried out.

Molecular modelling studies were performed using the published crystal structure of carboxypeptidase Y (protein data bank (PDB) 1YSC).^{8,9} The critical residues involved in the ester cleavage are: Ser146- involved in attack on the carbonyl of the ester and two glycine residues: Gly52 and Gly53 responsible for the appropriate coordination of the carbonyl group.

As can be seen in Figure 8.11, in the case of **8.8g-Sp** isomer, the stabilization by H-binding of two glycine residues is notable and the nucleophilic site of Ser146 is also well positioned (Figure 8.11).

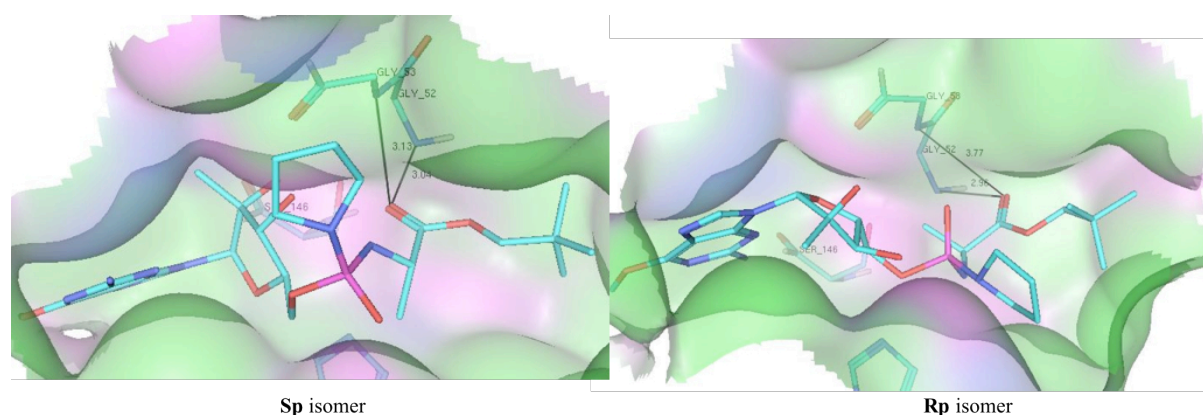


Figure 8.11. Docking studies of Sp and Rp isomers of **8.8g** into carboxypeptidase Y active site.

For the Rp isomer, the carbonyl group is situated a little further away from the Ser146 and therefore cannot efficiently interact with critical residues. These results support the difference in processing rate found for this compound during the enzymatic assay. It would be interesting to see whether the anti-HCV activity of separated isomers would differ as it has been seen in the case of PSI7977. As separation of diastereoisomers of **8.8g** was not possible, we were not able to conduct appropriate experiments.

On the basis of these differences we assumed that the more downfield species (one which was processed faster) in the ³¹P NMR spectra of **8.8g** corresponded to the Sp isomer. These findings would be in contrast with the data corresponding to the separated isomers of **INX-189**, where more downfield signal was represented by the Rp isomer.

8.9 Stability studies of 6-*O*-methyl-2'-*C*-methylguanosine symmetrical diamidates.

Given the very promising data of the new phosphorodiamidate prodrug motif, it was interesting to establish some stability outlines prior the animal pharmacokinetic studies. Therefore compound **8.2e** was incubated in the presence of mouse plasma, dog and human serum. Data are showing stability/decomposition of **8.2e** followed by the ^{31}P NMR over 12 h.

As can be seen in Figure 8.12, when parent phosphorodiamidate **8.2e** ($\delta_{\text{P}} = 14.50$ ppm) was incubated in the presence of mouse plasma at 37 °C, after 25 min only signal corresponding to the aminoacyl intermediate **5.21** (7.11 ppm) was observed.

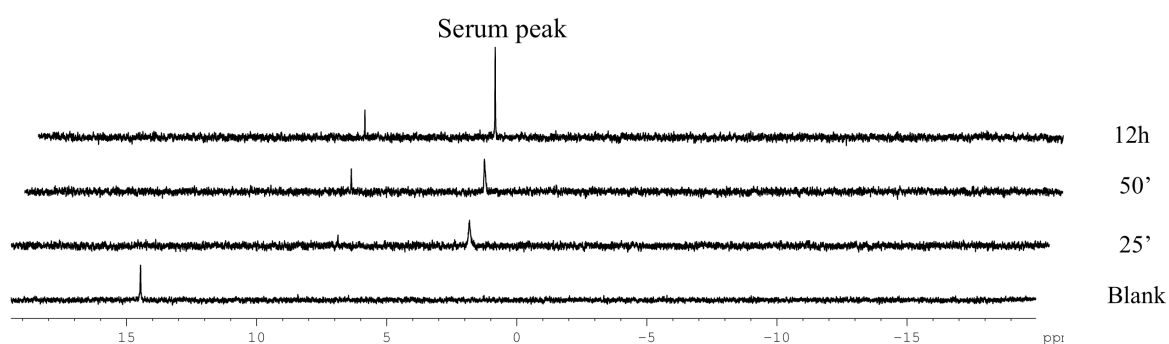


Figure 8.12. Stability of **8.2e** during the incubation with mouse plasma.

These data confirmed profound rodent plasma instability of **8.2e**, regardless the phosphorodiamidate motif. Reported data are consistent with stability data presented for aryloxyphosphoramidate ProTides described in Chapter 4, Section 4.8.1.

On the other hand when compound was incubated in the presence of either dog or human serum at 37 °C, **8.2e** was found to be completely stable and remained for the period of 12 h (Figure 8.13 and 8.14).

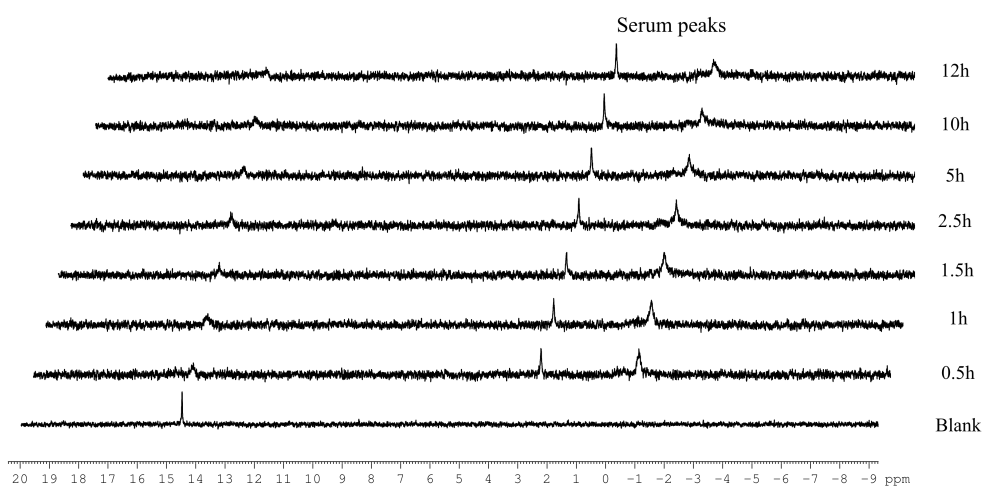


Figure 8.13. Stability of **8.2e** in dog serum.

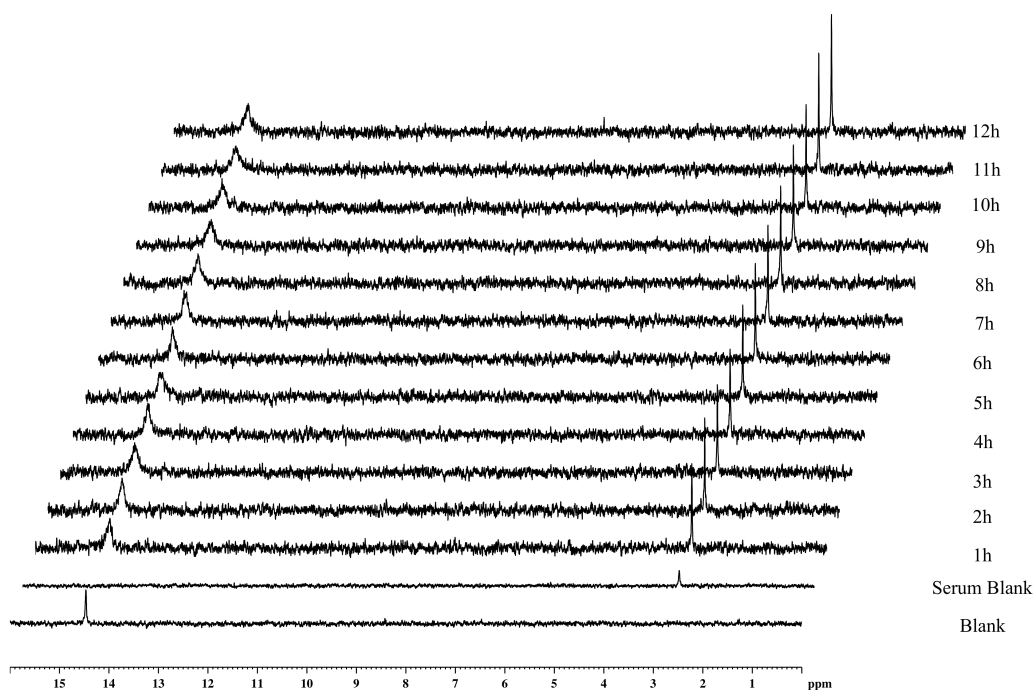


Figure 8.14. Stability of **8.2e** in human serum.

The phosphorodiamidate **8.2e** seems essentially stable in human serum, certainly for periods of hours appropriate for human dosing.

8.10 Intracellular levels of 2'-C-methylguanosine triphosphate – *in vitro* studies.

The initial investigation of 6-*O*-methyl-2'-*C*-methylguanosine phosphorodiamidates revealed promising means of 2'-*C*-methylguanosine 5'-monophosphate delivery into cells. In order to determine whether synthesised diamidates can be converted intracellularly to the active 2'-*C*-methylguanosine 5'-triphosphate, several diamidates were investigated in primary human hepatocytes. Studies were conducted and evaluated at Inhibitex Inc.

Phosphorodiamidates were incubated in primary human hepatocytes at arbitrary concentration of 10 μ M. Triphosphate levels were measured at 2 h, 6 h and 24 h time points using LC-MS/MS tandem spectroscopy. Additionally, levels of 2'CMeGTP obtain from the incubation of clinical candidate **INX-189** were measured alongside. Related data are reported in Figure 8.15.

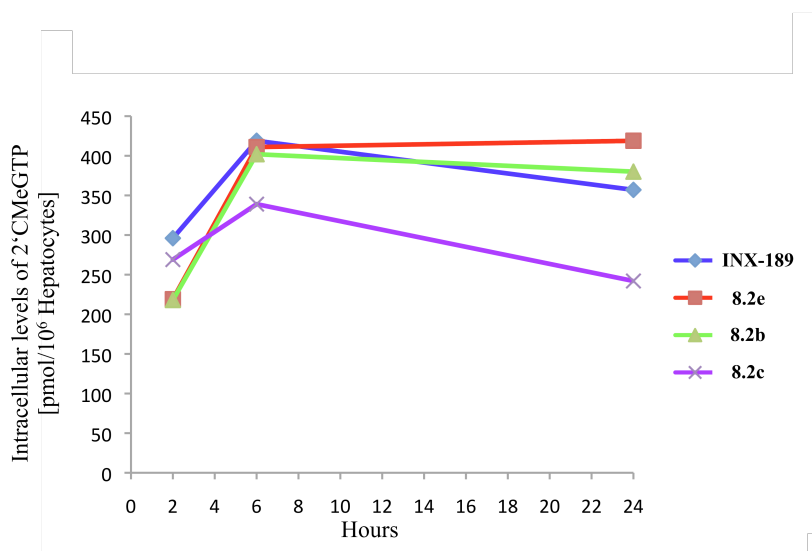


Figure 8.15. Levels of 2'CMeGTP produced from **INX-189**, **8.8b**, **8.8c** and **8.8e** in primary human hepatocytes system over 24 h incubation period.

As can be seen in Figure 8.15, incubation of all phosphorodiamidates in the presence of primary human hepatocytes resulted in the formation of high amounts of 2'-*C*-methylguanosine 5'-triphosphate. It can be clearly seen that the diamidate prodrugs were able to produce similar levels of the desired 5'-triphosphate as our lead compound **INX-189**. For compounds **8.2b** and **8.2c** the C_{\max} of TP was achieved within the first six hours of incubation and then slowly decayed. Additionally compound **8.2b** exhibit a very similar profile to **INX-189**, with C_{\max} of ca. 420 pmol/10⁶ cells. Interestingly the phosphorodiamidate analogue

of **INX-189** – **8.2e** was found to build up triphosphate levels up to 24 h and produce the highest overall triphosphate AUC across the tested compounds.

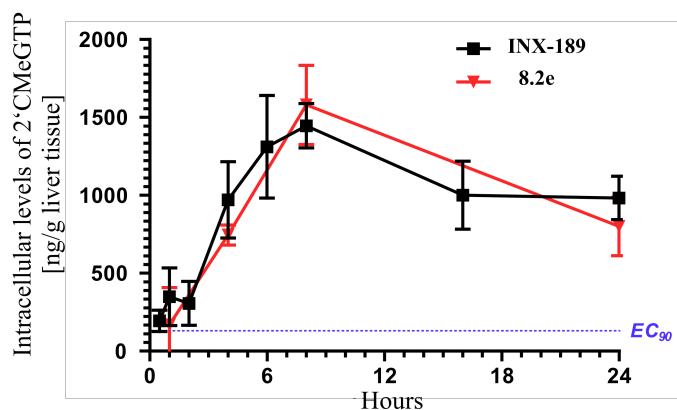
8.11 Intracellular levels of 2'-C-methylguanosine triphosphate – *in vivo* studies.

The ability of phosphorodiamidate **8.2e** to deliver monophosphate inside the liver cells with subsequent formation of the active 5'-triphosphate species after oral administration was investigated in a rat model.

The rat PK studies were performed by Inhibitex Inc.

The compound **8.2e** and **INX-189** were formulated in 95% Capmul MCM and 5% Tween 80, and administrated at 10 mg/kg dose as oral gavage to male Sprague-Dawley rats. Liver samples were collected at eight time points (up to 24 h post administration) and concentrations of 2'-C-methylguanosine triphosphate were measured by LC-MS/MS tandem spectroscopy. The related data are reported in the Figure 8.16.

The EC₉₀ value (243 pmol/g liver, 130.5 ng/g liver tissue) used as a reference in this study, was determined during the extensive evaluation of **INX-189** in the replicon cells, and has been described in Chapter 5 and 6.



EC₉₀: Defined as the amount of intra cellular triphosphate necessary to achieve 90% inhibition in the HCV replicon assay.

Figure 8.16. 2'-CMeGTP levels in livers of rats orally dosed with **INX-189** or **8.2e**.

It is clear that both of the compounds were able to produce significant levels of intracellular triphosphate in the rat liver after oral administration (Figure 8.16). Interestingly,

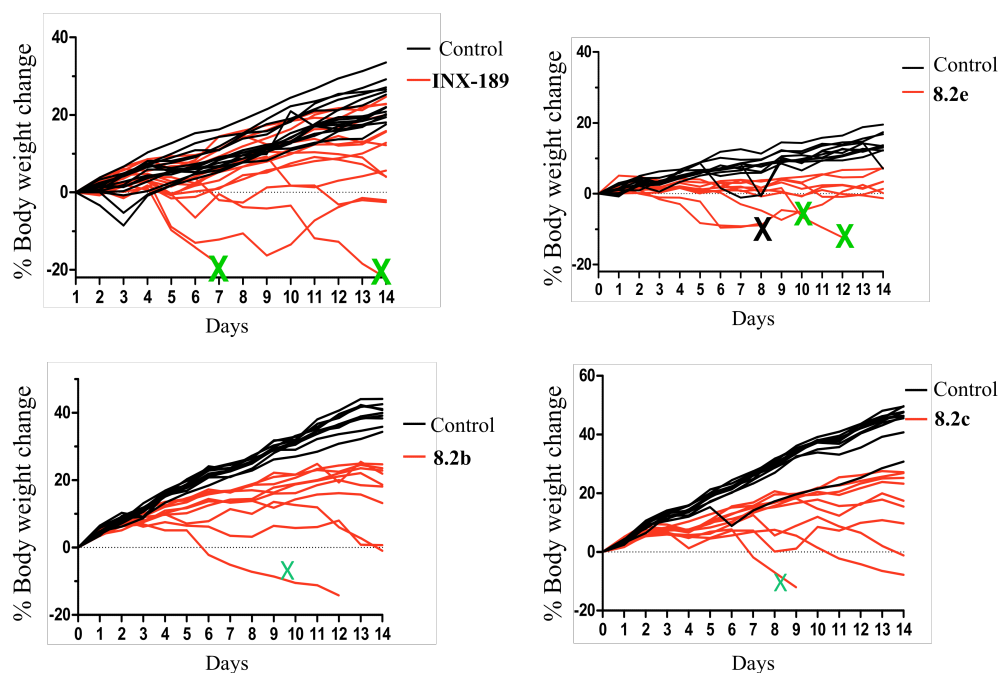
8.2e provide similar levels of the triphosphate as **INX-189** greatly exceeding the EC_{90} value by ca. 15-fold and maintained for over 24 h.

These data clearly show undisputed potential of the new prodrug motif and promise of potential phosphorodiamidate prodrug candidate suitable for the HCV clinical studies.

8.12 *In vivo* toxicity studies.

In order to determine the toxicity profile of the most interesting phosphorodiamidates (**8.8b**, **c** and **e**) and **INX-189**, compounds were evaluated in 14-days rat toxicity studies at Inhibitex Inc.

Compounds were formulated in the standard PO formulation (95% Capmul MCM and 5% Tween 80) and were orally dosed to male Sprague-Dawley rats at 30 mg/kg once daily for 14 days. The body weight was checked daily pre-dosing. All tested groups were checked against vehicle group, which was orally dosed with 95% Capmul MCM and 5% Tween 80 only. Obtained data are presented in Figure 8.17.



✕- Necropsied in moribund state, ✕- Found dead.

Figure 8.17. 14-Days rat toxicity studies.

As reported in Figure 8.17, all tested phosphorodiamidates **8.2b**, **c** and **e** showed favourable toxicity profile comparing to the **INX-189**, with less variation in the body mass comparing to the vehicle groups.

In the case of **8.2e** there was one case of mortality and in each treated groups 1 to 2 animals were found in moribund condition. Upon necropsy, the moribund animals showed signs of gastrointestinal tract toxicity namely haemorrhaging in intestines. Apart from that no other signs of toxicity or any other adverse events were observed.

8.13 Conclusions.

In conclusion, a series of novel 6-*O*-methyl-2'-*C*-methylguanosine phosphorodiamidates derived from different amino acids and/or simple amines has been synthesised. This type of 5'-monophosphate prodrugs as shown in this Chapter, can be designed to be achiral or chiral at the phosphate centre if desired. During the extensive structure activity relationship studies, compounds exhibiting nM activity against HCV replicon assay were identified. In general phosphorodiamidates were not toxic in the cell-based assay up to 100 μ M concentration. This is the first time, when application of a phosphorodiamidate approach to the nucleoside analogue is successful. Carboxypeptidase Y studies revealed that the cleavage of only one of the ester moieties is essential for the efficient activation of diamidates and their potent activity versus the hepatitis C virus. Additionally phosphorodiamidates were found to be stable in dog and human serum. All tested diamidates showed ability to produce substantial levels of 2'-*C*-methylguanosine 5'-triphosphate, similar to the one exhibited by **INX-189**. Several compounds were advanced into the rat PK studies, and it was demonstrated that phosphorodiamidates could provide high levels of 2'CMeGTP *in vivo* in rat livers.

The combination of replicon activity with *in vitro* and *in vivo* data resulted in the identification of several compounds, suitable for **INX-189** back up programme.

References:

1. Jones BCNM, McGuigan C, O'Connor TJ, Jeffries DJ, Kinchington D. Synthesis and anti-HIV activity of some novel phosphorodiamidate derivatives of 3'-azido-3'-deoxythymidine (AZT). *Aniviral Chem Chemother* **1991**, 2, 35-39.
2. Phelps ME, Woodman PW, Danenberg PV. Synthesis and biological activity of 5'-fluoro-2'-deoxyuridine 5'-phosphorodiamidates. *J Med Chem* **1980**, 23, 1229-1232.
3. McGuigan C, Jones BCNM, Devine KD, Nicholls SR, O'Connor TJ, Kinchington D. Attempts to introduce chemotherapeutic nucleotides into cells: studies on the anti-HIV agent FdT. *Bioorg Med Chem Lett* **1991**, 12, 729-732.
4. Serafinowska HT, Ashton RJ, Bailey S, Harnden MR, Jackson SM, Sutton D. Synthesis and in vivo evaluation of prodrugs of 9-[2-(phosphonomethoxy)ethoxy]adenine. *J Med Chem* **1995**, 38, 1372-1379.
5. Keith KA, Hitchcock MJ, Lee WA, Holy A, Kern ER. Evaluation of nucleoside phosphonates and their analogues and prodrugs for inhibition of orthopoxvirus replication. *Antimicrob Agents Chemother* **2003**, 47, 2193-2198.
6. Yoshikawa M, Kato T, Takenishi T. A novel method for phosphorylation of nucleosides to 5'-nucleotides. *Tetrahedron Lett* **1967**, 5065-5068.
7. McGuigan C, Madela K, Aljarah M, Bourdin C, Arrica M, Barrett M, Jones S, Kolykhalov A, Bleiman B, Bryant KD, Ganguly B, Gorovits E, Henson G, Hunley D, Hutchins J, Muhammad J, Obikhod A, Patti J, Walters CR, Wang J, Vernachio J, Ramamurty CVS, Battina SK, Chamberlain S. Phosphoramidates as promising new phosphate prodrug motif for antiviral drug discovery: Application to anti-HCV agents. *J Med Chem* **2011**, 54, 8632-8645.
8. Endrizzi JA, Breddam K, Remington SJ. 2.8-A Structure of yeast serine carboxypeptidase. *Biochemistry* **1999**, 33, 11106-11120.
9. Jung G, Ueno H, Hayashi R. Carboxypeptidase Y: structural basis for protein sorting and catalytic triad. *J. Biochem.* **1999**, 126, 1-6.

CHAPTER NINE

In search of new pharmacophore: C-8 modifications.

9.1 8-C-modified 6-*O*-methyl-2'-*C*- β -methylguanosine – the rational behind the design.

Recently 8-halogenated purine analogues have been recognized as an interesting class of biologically active agents. 8-Chloroadenosine (8ClAdo), a dephosphorylated metabolite of 8-chloro-cyclicAMP has been involved in pre-clinical and clinical studies for the treatment of human multiple myeloma, human promyelocytic leukemia and solid tumors.¹ 8ClAdo is cytotoxic to cell lines that are resistant to other drugs and therefore it can constitute as an alternative for the treatment of neoplastic diseases.²

Although, the effect of the C8-modified purine nucleosides has been well studied in the field of anti-cancer agents little is known about the antiviral activity of these type of molecules.

To address the effect of C8 steric bulk on the anti-HCV activity, family of analogues of 6-*O*-methyl-2'-*C*-methylguanosine that contained halogens of increasing size (chlorine, bromine and iodine) at the C8-position was synthesised.

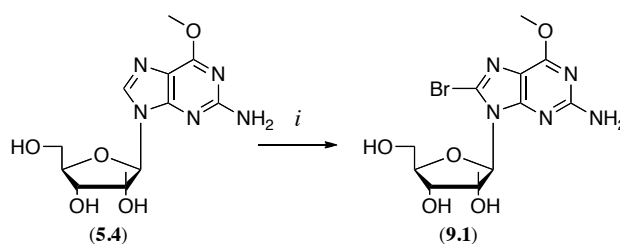
Introduction of a substituent in C-8 position (in contrast to the most of the modifications in C-2 or C-6 position) preserves the pairing with pyrimidines and the ability to form nucleic acid duplexes in which the substituent would point out to the major groove and thus modulate the interactions with proteins.⁴

9.2 Synthesis of 8-C-halo-6-modified-2'-*C*- β -methylguanosine nucleoside analogues.

9.2.1 Synthesis of 8-C-bromo-6-*O*-methyl-2'-*C*- β -methylguanosine.

The synthesis of 8-*C*-bromo-2'-deoxyguanosine from 2'-deoxyguanosine in the presence of *N*-bromosuccinamide in water has been reported for the first time by Gannett and Sura in 1993.⁵ We decided to investigate this procedure in order to synthesise 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine. The trial reaction in the exact conditions as described by Gannett *et al.* did not result in product formation. The main issue was the solubility of the

starting material. Examination of different solvent systems, provided the best results. When dry methanol was used as a sole solvent and 1 mol equivalent of *N*-bromosuccinamide (NBS) as brominating agent, at ambient temperature for 4 hours (Scheme 9.1), after purification on silica gel, using CHCl₃ : MeOH (0 to 4% gradient) as an eluent, pure compound was obtained as a white solid in 88% yield.



Reagents and Conditions: i) NBS, dry MeOH, rt, 4 h.

Scheme 9.1. Synthesis of 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine.

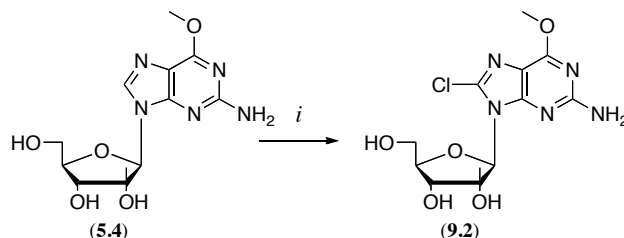
9.2.2 Synthesis of 8-*C*-chloro-6-*O*-methyl-2'-*C*-β-methylguanosine.

There are several reported methods for chlorination at the C8-position of purines, including reaction with benzoyl chloride (BzCl) and *m*-chlorobenzoic acid (*m*CPBA).⁶ Although this method was very successful in the preparation of 8-chloroadenosine, it proved to be not effective with the guanosine analogue (Table 9.1). Application of the 8-bromo analogue methodology, using *N*-chlorosuccinamide (NCS) did not provide the desired product. Increase of the reaction time and temperature had no impact on the product formation (Table 9.1).

| Entry | Starting material | Reagents | Conditions | Yield % |
|-------|-------------------|--|-------------|---------|
| 1 | 5.4 (1 mol eq) | BzCl (1.1 mol eq) <i>m</i> CPBA (1.1 mol eq) dry DMF | rt, 20 min | - |
| 2 | 5.4 (1 mol eq) | NCS (1.0 mol eq) dry MeOH | rt, 4 h | - |
| 3 | 5.4 (1 mol eq) | NCS (2.0 mol eq) dry MeOH | rt, 18 h | - |
| 4 | 5.4 (1 mol eq) | NCS (2.0 mol eq) dry MeOH | 50 °C, 18 h | - |

Table 9.1. Attempts (reagents and conditions) to synthesise 8-*C*-chloro-6-*O*-methyl-2'-*C*-methylguanosine.

After several unsuccessful attempts, the procedure reported by Hamm *et al.* was investigated.⁷ Thus, 6-*O*-methyl-2'-*C*-methylguanosine **5.4** was reacted with NCS in dry THF at 35 °C, for 16 h (Scheme 9.2).



Reagents and Conditions: i) NCS, dry THF, 35 °C, 16 h.

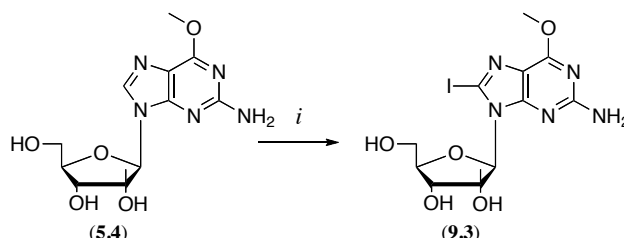
Scheme 9.2. Synthesis of 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine.

After purification on silica gel, using CHCl₃ : MeOH (0 to 4% gradient) as an eluent, pure compound was obtained as a white solid in an excellent 85% yield.

9.2.3 Synthesis of 8-*C*-iodo-6-*O*-methyl-2'-*C*-β-methylguanosine.

In the first attempt to synthesise 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine, reaction was carried out in dry methanol, in the presence of *N*-iodosuccinamide (NIS) at ambient temperature. However the reaction resulted in a complex mixture of potential products, which proved to be difficult to isolate.

Lipkin *et al.* reported successful iodination of guanine in dimethyl sulfoxide.⁸ A drawback of this protocol is the use of DMSO and troublesome isolation of the product from the high boiling-point solvent and from unreacted starting material. Since the iodination procedure was similar to the well established route to the 8-*C*-chloro-6-*O*-methyl-2'-*C*-methylguanosine, the method of Hamm *et al.* was applied (Scheme 9.3).



Reagents and Conditions: i) NIS, dry THF, 35 °C, 3 days.

Scheme 9.3. Synthesis of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine.

The extension of reaction time to 3-days, resulted in formation of the desired product, which after purification was obtained as a yellowish solid in 51% yield.

The introduction of the halogen atom onto C8-position can be easily recognized by a chemical shift of the C-8 carbon atom (Figure 9.1 and 9.2).

In the parent nucleoside **5.4**, the C-8 carbon is represented by a single negative signal at 139.35 ppm in the ^{13}C PENDANT spectrum (Figure 9.1).

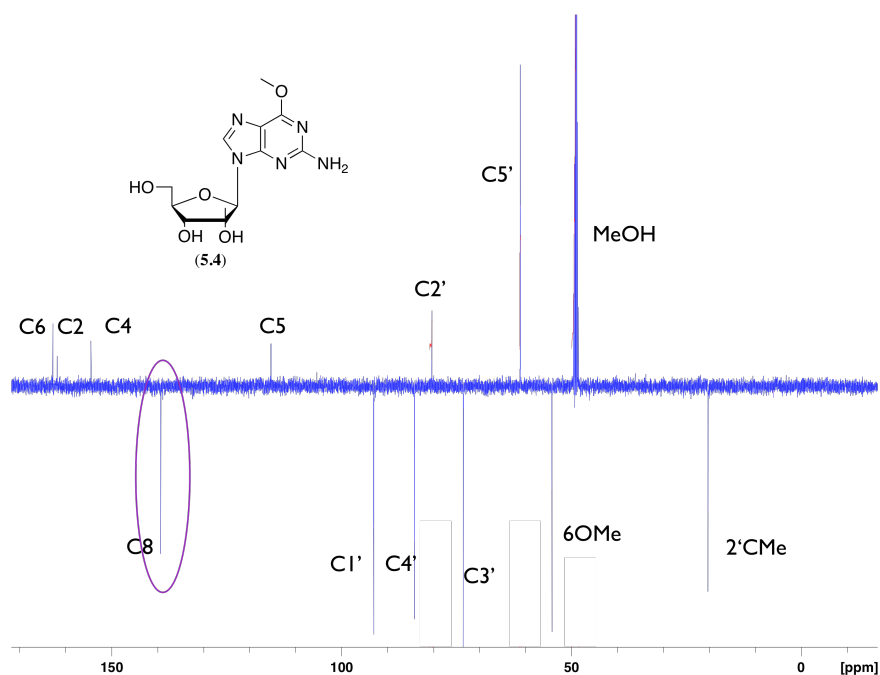
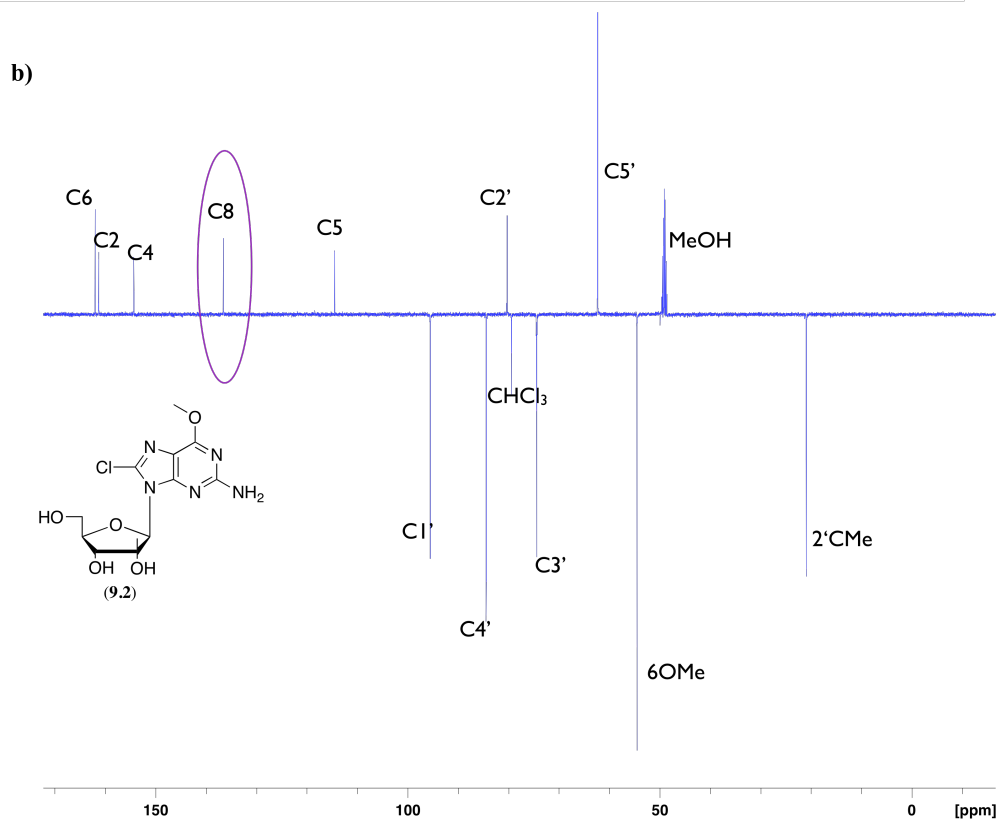
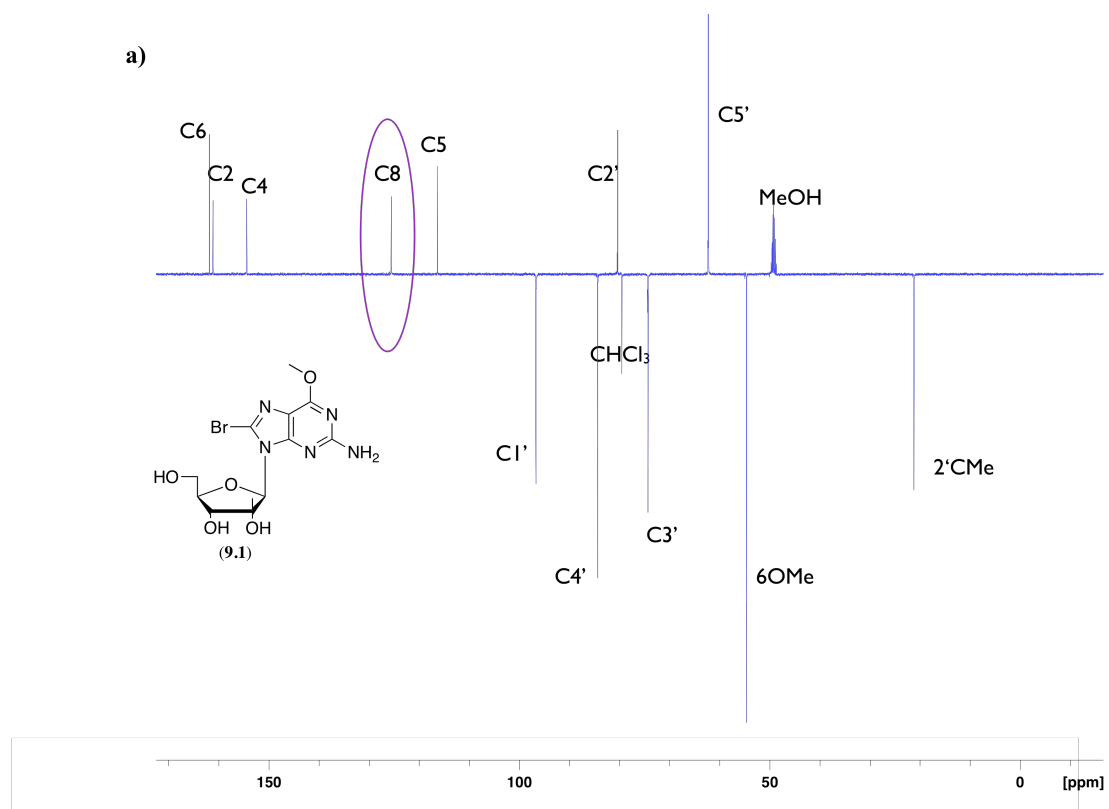


Figure 9.1. The ^{13}C PENDANT spectrum of 6-*O*-methyl-2'-*C*-methylguanosine.

Introduction of bromine atom resulted in the appearance of more upfield positive signal at $\delta_{\text{C}} = 125.57$ ppm (Figure 9.2a). Replacement of C8-H by the chlorine atom gave ^{13}C peak at 136.59 ppm (Figure 9.2b) with only ca. 2 ppm difference comparing to the parent nucleoside **5.4**. From all halogens introduced onto C-8 position, the biggest effect was observed for the iodine atom. The peak of C-8 observed for **9.3** was shifted ca. 40 ppm more upfield comparing to the starting material **5.4**, resulting in a positive signal at $\delta_{\text{C}} = 99.27$ ppm.



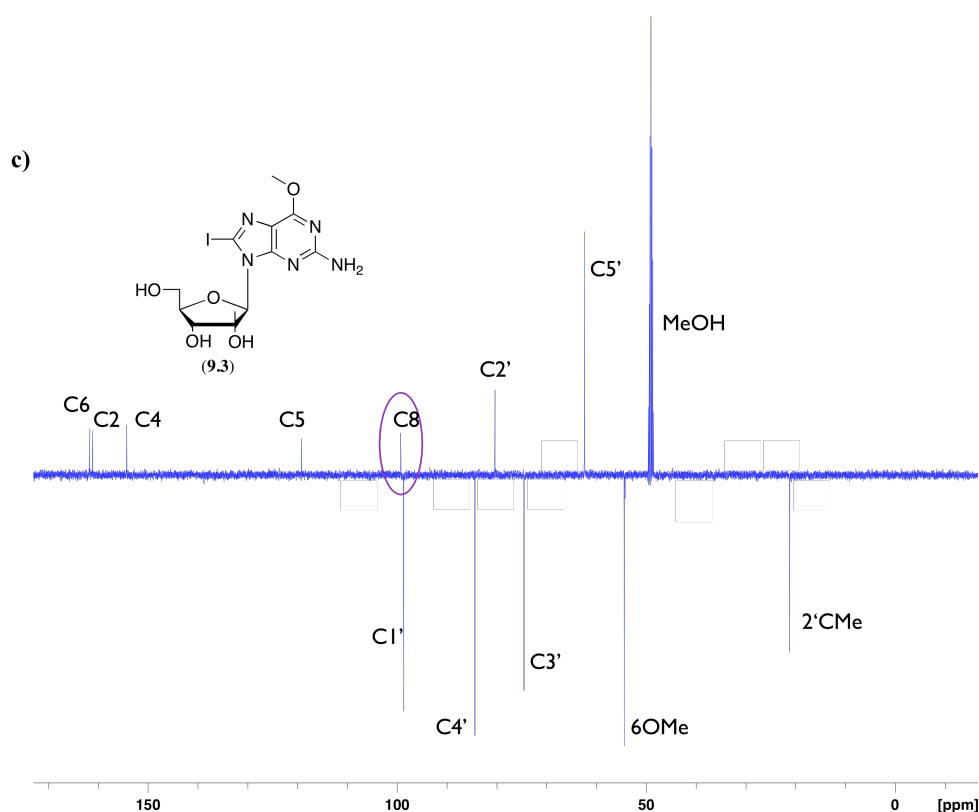


Figure 9.2. ^{13}C PENDANT spectra of a) **9.1**; b) **9.2**; c) **9.3**.

The upfield trend in the ^{13}C chemical shift, observed for the compounds **9.2** and **9.3**, is caused by the bromine and iodine atom, and is called the ‘heavy atom effect’. This is attributed to the increased diamagnetic shielding caused by the large number of electrons introduced by heavy atoms.⁹

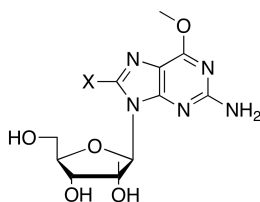
All newly synthesised nucleosides were more lipophilic than the parent nucleoside **5.4** (Table 9.2).

| Cpd | C8-substituent | Yield % | C8 ^{13}C NMR [δ ppm] MeOD | ClogP |
|------------|----------------|---------|---|-------|
| 5.4 | H | - | 139.35 | -0.6 |
| 9.1 | Br | 88 | 125.57 | 0.2 |
| 9.2 | Cl | 85 | 136.59 | 0.04 |
| 9.3 | I | 51 | 99.27 | 0.2 |

Table 9.2. Summary of C8-modified nucleoside analogues synthesised, reaction yields, C8 ^{13}C PENDANT NMR shifts and calculated lipophilicity.

9.2.4 Biological evaluation of 8-C-halo-6-modified-2'-C- β -methylguanosine nucleoside analogues.

All novel C8-modified analogues of 6-*O*-methyl-2'-*C*-methylguanosine were evaluated for their anti-HCV activity using *in vitro* replicon assay by Inhibitex Inc (Table 9.3).



| Cpd | X | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|-----|----|-------|-----------------------|-----------------------|
| 5.4 | H | -0.6 | 4.6 | >100 |
| 9.1 | Br | 0.2 | >100 | >100 |
| 9.2 | Cl | 0.04 | >100 | >100 |
| 9.3 | I | 0.2 | 6.0 | >100 |

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%;
CC₅₀: 50% cytotoxic concentration.

Table 9.3. Biological activity and calculated lipophilicity of C-8 modified analogues of 6-*O*-methyl-2'-*C*-methylguanosine.

As can be seen in Table 9.3, the analogues bearing the bromo- and chloro-substituents in the C8-position (**9.1** and **9.2**, respectively), were found to be completely inactive in the replicon assay EC₅₀ > 100 μM. On the other hand C8-iodo analogue **9.3** was found to be as potent as 6-*O*-methyl-2'-*C*-methylguanosine **5.4** (EC₅₀ = 6.0 μM vs. 4.6 μM). This result was somewhat surprising as none of the smaller size halogens provided any biological activity. These results evidently indicate the importance of the iodine substitution in C8-position however it was not clear whether this particular halogen is a part of a new pharmacophore or is being metabolized on one of the steps during activation to the triphosphate species.

The phosphoramidate technology was applied to the nucleoside analogues **9.1-9.3** in order to investigate whether their *in vitro* anti-HCV activity can be enhanced. Synthesis and biological evaluation of aryloxyphosphoramidates of **9.1-9.3** will be described in Section 9.3.

9.2.5 Adenosine deaminase assay on 8-*C*-iodo-6-*O*-methyl-2'-*C*- β -methylguanosine.

In order to investigate whether the 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine can serve as a suitable substrate for the adenosine deaminase mediated hydrolysis; enzymatic assay in the presence of ADA was conducted, according to the procedure described in Chapter 5, Section 5.2.3.

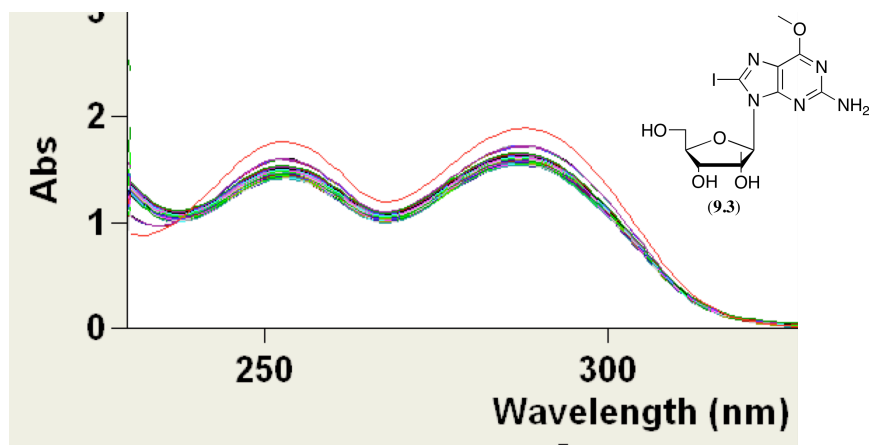


Figure 9.3. Spectral change during ADA-mediated hydrolytic removal of alkyl group from **9.3**.

As can be seen in Figure 9.3, nucleoside analogue **9.3** was found to be completely stable during 16 h course of the experiment, showing that **9.3** cannot serve as a substrate for ADA. The presence of the iodine in the C8-position prevents the enzyme mediated hydrolysis of the C6 alkyl substituent. This profound lack of activity may be related to the conformation of the nucleoside. It is very likely that the C8-iodo analogue can preferentially adopt *syn* conformation, wherein the nucleobase is positioned over the ribose sugar. This conformation, which is in contrast to the *anti* conformation of ordinary purine nucleosides, arises because the steric bulk of the iodine precludes its residence over the ribose ring.

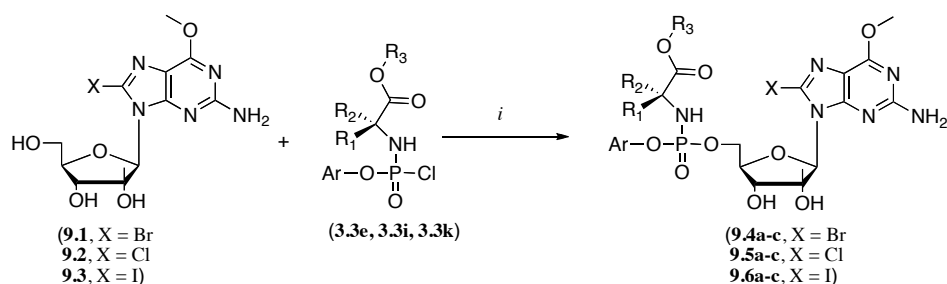
Substrate specificity of AMPDA suggests that the 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine 5'-monophosphate could not serve as a suitable substrate for the enzyme mediated hydrolysis of the C6-substituent. However it is not clear, whether ADAL1 could accept the presence of the bulky substituent in the C-8 position of the modified nucleoside and perform hydrolytic cleavage of the methoxy moiety in order to obtain C6-keto analogue.

9.3 Synthesis of 8-*C*-halo-6-*O*-modified-2'-*C*- β -methylguanosine prodrugs.

9.3.1 Synthesis of a first series of 8-*C*-halo-6-*O*-modified-2'-*C*- β -methylguanosine prodrugs.

The aryloxyphosphoramidate approach was applied to the 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine analogues **9.1-9.3** in order to investigate the influence of ProTide technology on anti-HCV activity of these nucleoside analogues. To facilitate the comparison all compounds were prepared as α -naphthyl-L-alanine analogues bearing two different esters: 2,2-dimethylpropyl and benzyl, and also as α -naphthyl-L-valine-*O*-benzyl derivatives.

All compounds were synthesised using *t*BuMgCl mediated coupling reaction, described in Chapter 4 and 5 (Scheme 9.4, Table 9.4).



Reagents and Conditions: *i*) *t*BuMgCl, dry THF, rt, 16 h.

Scheme 9.4. Synthesis of aryloxyphosphoramidates of 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine.

| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | yield % |
|-------------|----|----------------|-----------------------------------|----------------|-------|------------------------|-------|---------|
| 9.4a | Br | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.2 | 23 |
| 9.4b | Br | α -Naph | CH ₃ | H | L-Ala | benzyl | 4.0 | 13 |
| 9.4c | Br | α -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.9 | 26 |
| 9.5a | Cl | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.0 | 23 |
| 9.5b | Cl | α -Naph | CH ₃ | H | L-Ala | benzyl | 3.8 | 16 |
| 9.5c | Cl | α -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.8 | 17 |

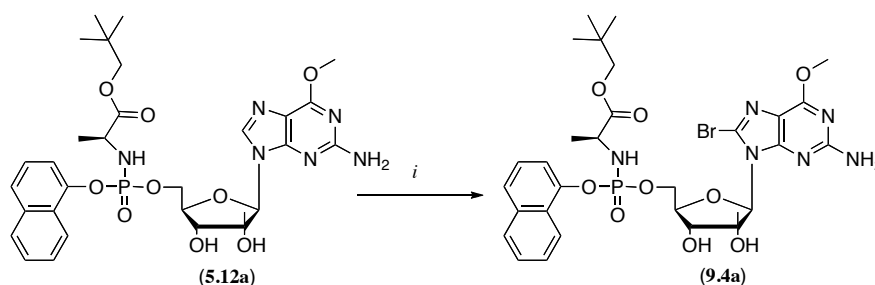
| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | yield % |
|-------------|---|----------------|-----------------------------------|----------------|-------|------------------------|-------|---------|
| 9.6a | I | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.1 | 27 |
| 9.6b | I | α -Naph | CH ₃ | H | L-Ala | benzyl | 3.9 | 14 |
| 9.6c | I | α -Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.9 | 13 |

AA-amino acid, ClogP values calculated using ChemOffice ultra 11.0.

Table 9.4. Summary of a first series of 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine ProTides, their calculated lipophilicity and reaction yields.

All novel ProTides were obtained as white solids in moderate to good yields (13-27%). Compounds were isolated as roughly 1:1 mixtures of phosphate diastereoisomers as evidenced by ³¹P NMR and HPLC.

From the synthetic point of view, it was of interest to investigate whether the introduction of the halogen atom would be possible at the ProTide level. To probe this, aryloxyphosphoramidate **5.12a** was dissolved in dry methanol and treated with 1 mol equivalent of *N*-bromosuccinamide in order to obtain compound **9.4a** (Scheme 9.5).



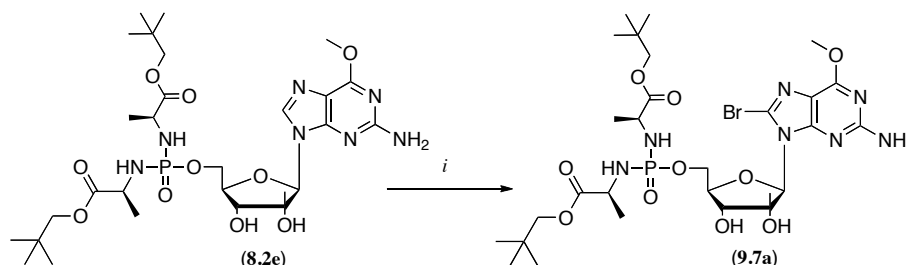
Reagents and Conditions: i) NBS, dry MeOH, rt, 4 h.

Scheme 9.5. Synthesis of aryloxyphosphoramidates of 8-*C*-bromo modified ProTides of 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine.

The bromination reaction was clean and resulted in a formation of **9.4a** in excellent 94% yield during four hours reaction course. Comparing two possible routes of synthesis of 8-*C*-bromo modified ProTides it was noticed that the second route (bromination on the ProTide level) was slightly better in terms of overall yield (25% vs. 20%). Not much difference in terms of purification between two synthetic pathways was observed.

However when **5.12a** was treated with either *N*-chlorosuccinamide or *N*-iodosuccinamide in reaction conditions described above, no product formation was observed.

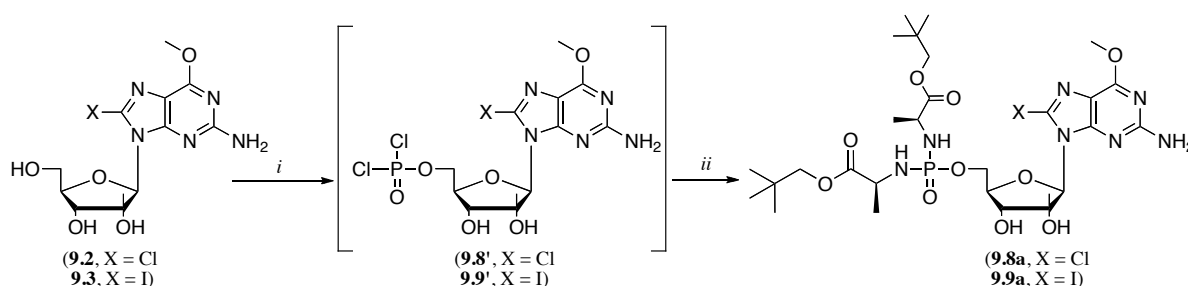
Additionally we decided to investigate the effect of phosphorodiamidate motif on the anti-HCV activity of the 8-modified nucleosides **9.1-9.3**. In the first attempt phosphorodiamidate **8.2e** was reacted with NBS in dry methanol for 6 h (Scheme 9.6).



Reagents and Conditions: i) NBS, dry MeOH, rt, 6 h.

Scheme 9.6. Synthesis of phosphorodiamidate **9.7a** via direct NBS bromination.

The pure compound **9.7a** was obtained in 50% yield after purification on silica gel, using CHCl_3 : MeOH (97:3) as an eluent. Similar attempt performed with NCS or NIS did not result in product formation. In order to obtain 8-*C*-chloro- and 8-*C*-iodo phosphorodiamidate analogues of **9.7a**, synthetic procedure used for the synthesis of 6-*O*-methyl-2'-*C*-methylguanosine diamidates was applied (Scheme 9.7).



Reagents and Conditions: i) POCl_3 , Et_3N , dry THF, $-78\text{ }^\circ\text{C}$ to rt, 30 min; ii) L-alanine-O-2,2-dimethylpropyl ester x *p*TSA, Et_3N , DCM, $-78\text{ }^\circ\text{C}$ to rt, overnight.

Scheme 9.7. Synthesis of symmetrical phosphorodiamidates of **9.2** and **9.3** via POCl_3 route.

After column chromatography, compounds **9.8a** and **9.9a** were obtained as white solids in 26% and 10% yield, respectively (Table 9.5).

| Cpd | X | AA | R ₃ (ester) | Synthetic route | ClogP | yield % |
|------|----|-------|------------------------|-------------------|-------|---------|
| 9.7a | Br | L-Ala | 2,2-dimethylpropyl | NBS | 5.1 | 50 |
| 9.8a | Cl | L-Ala | 2,2-dimethylpropyl | POCl ₃ | 4.9 | 26 |
| 9.9a | I | L-Ala | 2,2-dimethylpropyl | POCl ₃ | 5.0 | 10 |

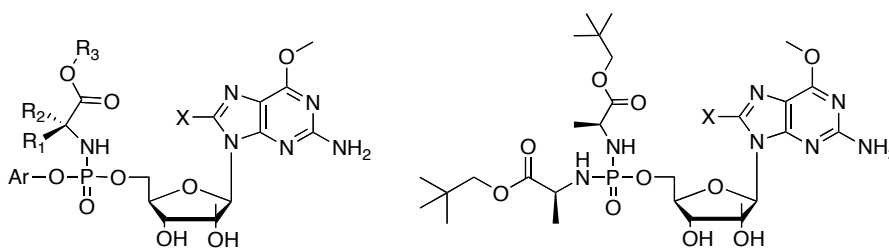
AA-amino acid, ClogP values calculated using ChemOffice ultra 11.0.

Table 9.5. Summary of a first series of 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine diamidates, their calculated lipophilicity and reaction yields.

At this point it was clear that only 8-*C*-bromo analogues could be synthesised via NBS-mediated direct bromination of either phosphoramidate or phosphordiamidate prodrugs, synthesis of 8-*C*-chloro or 8-*C*-iodo analogues required coupling reactions of C8-modified nucleosides with the appropriate phosphorochloridates or POCl₃ (followed by the addition of amino acid ester).

9.3.2 Biological evaluation of a first series of 8-*C*-halo-6-*O*-modified-2'-*C*-β-methylguanosine prodrugs.

All newly synthesised prodrugs of 8-*C*-halo-6-*O*-modified-2'-*C*-methylguanosine analogues were evaluated for their ability to inhibit viral replication in *in vitro* replicon assay by Inhibitex Inc (Table 9.6).



| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|---------|----|--------|-----------------------------------|----------------|-------|------------------------|-------|-----------------------|-----------------------|
| INX-189 | H | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3.3 | 0.01 | 7 |
| 9.1 | Br | - | - | - | - | - | 0.2 | >100 | >100 |
| 9.4a | Br | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.2 | 14 | 60 |
| 9.4b | Br | α-Naph | CH ₃ | H | L-Ala | benzyl | 4.0 | 16 | >100 |
| 9.4c | Br | α-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.9 | 9 | >30 |
| 9.2 | Cl | - | - | - | - | - | 0.04 | >100 | >100 |
| 9.5a | Cl | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.0 | >10 | 59 |
| 9.5b | Cl | α-Naph | CH ₃ | H | L-Ala | benzyl | 3.8 | >40 | 83 |

| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|-------------|---------------------|--------|-----------------------------------|----------------|-------|------------------------|-------|-----------------------|-----------------------|
| 9.5c | Cl | α-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.8 | 10 | 25 |
| 9.3 | H | - | - | - | - | - | 0.2 | 6.0 | >100 |
| 9.6a | I | α-Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.1 | 0.02 | 14 |
| 9.6b | I | α-Naph | CH ₃ | H | L-Ala | benzyl | 3.9 | 0.04 | 18 |
| 9.6c | I | α-Naph | CH(CH ₃) ₂ | H | L-Val | benzyl | 4.9 | 0.30 | 25 |
| Cpd | Phosphorodiamidates | | | | | | | | |
| 9.7a | Br | - | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 5.1 | >40 | >100 |
| 9.8a | Cl | - | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 4.9 | >10 | >100 |
| 9.9a | I | - | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 5.0 | 0.13 | 54 |

AA: Amino acid, EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 9.6. Summary of the anti-HCV activity of 8-*C*-modified 6OMe2'CMcG phosphoramidates and diamidates, parent nucleosides **9.1-9.3**, **INX-189**, and their calculated ClogP values.

As can be seen in Table 9.6, in the case of 8-*C*-bromo (**9.4a-c**, **9.7a**) and 8-*C*-chloro (**9.5a-c**, **9.8a**) analogues the application of aryloxyphosphoramidate or phosphorodiamidate technology did not improve the anti-HCV activity of the corresponding parent nucleosides **9.1** and **9.2**, respectively. The explanation for this lack of activity might be that these nucleoside analogues are not capable to interact with the viral polymerase and therefore cannot cause inhibition of viral replication or that one or more steps in the putative activation mechanism is blocked. On the other hand, all analogues bearing C8-iodo modification exhibit *in vitro* anti-HCV activity in submicromolar range. Application of the aryloxyphosphoramidate or diamidate technology resulted in enhancement of antiviral activity up to 300-fold. The most potent derivative across this set of compounds was **9.6a** (EC₅₀ = 0.02 μM), with activity comparable to that of the clinical candidate **INX-189** (EC₅₀ = 0.01 μM).

It has been noticed that the L-alanine derivative **9.6b** was ca. 8-times more active than the corresponding L-valine analogue **9.6c** (EC₅₀ = 0.04 μM vs. EC₅₀ = 0.30 μM, respectively). This observation is in agreement with the previous data obtain for 2'CMcG and 6OMe2'CMcG ProTides (Chapter 4 and 5), showing superior role of L-alanine over L-valine. Additionally it was found that aryloxyphosphoramidate produgs were slightly more potent than the analogous diamidates (**9.6a** vs. **9.9a**, EC₅₀ = 0.02 μM vs. EC₅₀ = 0.13 μM, respectively).

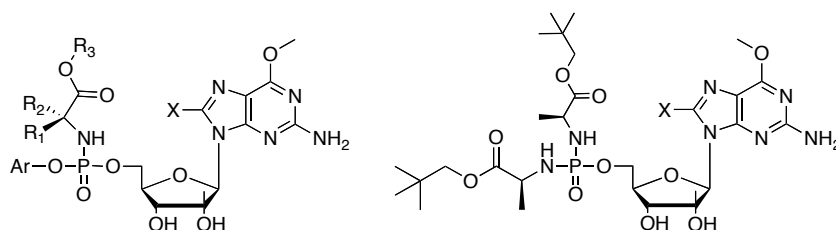
In general compounds 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine prodrugs were found to exhibit some toxicity in the cell-based assay (at μM level), however their high potency still leads to the therapeutic index values in the 1000 range.

Given the extremely high potency of the **9.6a** derivative we decided to vary the ProTide and diamidate motives on 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine.

9.3.3 SAR studies of a second series of 8-*C*-iodo-6-*O*-modified-2'-*C*- β -methylguanosine prodrugs.

The second family of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine prodrugs was obtained following the procedures as in Scheme 9.4 and 9.7, but varying the phosphorochloridate and/or amino acid esters. All synthesised compounds (except diamidate prodrugs) were isolated as roughly 1:1 diastereomeric mixtures at the phosphorus centre, in moderate to good yields (14-30 % for phosphoramidates and 23-40% for diamidates).

Compounds were evaluated for their inhibitory activity in replicon based assay (Inhibitex Inc.) and the corresponding results are reported in Table 9.7.



| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|-------------|---|----------------|-----------------|----------------|-------|------------------------|-------|------------------------------------|------------------------------------|
| INX-189 | H | α -Naph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 3.3 | 0.01 | 7 |
| 9.3 | I | - | - | - | - | - | 0.2 | 6.0 | >100 |
| 9.6d | I | α -Naph | CH ₃ | H | L-Ala | <i>n</i> -propyl | 3.3 | 0.0074 | 6.1 |
| 9.6e | I | α -Naph | CH ₃ | H | L-Ala | <i>n</i> -butyl | 3.8 | 0.011 | 7.9 |
| 9.6f | I | α -Naph | CH ₃ | H | L-Ala | <i>n</i> -pentyl | 4.4 | 0.012 | 11 |
| 9.6g | I | α -Naph | CH ₃ | H | L-Ala | cyclobutyl | 3.2 | 0.010 | 6.9 |
| 9.6h | I | α -Naph | CH ₃ | H | L-Ala | cyclopentyl | 3.7 | 0.012 | 59 |
| 9.6i | I | α -Naph | CH ₃ | H | L-Ala | cyclohexyl | 4.3 | 0.016 | 11 |
| 9.6j | I | α -Naph | CH ₃ | H | L-Ala | tetrahydropyranyl | 1.9 | 0.018 | 26 |

| Cpd | X | Ar | R ₁ | R ₂ | AA | R ₃ (ester) | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|----------------------------|---|--------|--|----------------|-------|------------------------|-------|-----------------------|-----------------------|
| 9.6k | I | α-Naph | CH ₃ | H | L-Ala | 2,4-diF-benzyl | 4.2 | 0.010 | 8.1 |
| 9.6l | I | Ph | CH ₃ | H | L-Ala | 2,2-dimethylpropyl | 2.9 | 0.03 | 17 |
| 9.6m | I | α-Naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | <i>i</i> -propyl | 3.9 | 0.07 | 18 |
| 9.6n | I | α-Naph | CH ₂ CH ₂ SCH ₃ | H | L-Met | benzyl | 4.9 | 0.034 | 25 |
| Phosphorodiamidates | | | | | | | | | |
| 9.9b | I | - | CH ₃ | H | L-Ala | 3,3-dimethyl-1-butyl | 6.1 | 0.072 | 11 |
| 9.9c | I | - | CH ₃ | H | L-Ala | cyclohexyl | 5.4 | 0.13 | 54 |
| 9.9d | I | - | CH ₃ | H | L-Ala | benzyl | 4.8 | 0.046 | 22 |

AA: Amino acid, EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 9.7. Summary of the anti-HCV activity of 8-*C*-iodo-6OMe2'CMeG phosphoramidates and diamidates, parent nucleoside **9.3**, **INX-189**, and their calculated ClogP values.

All synthesised prodrugs displayed improved EC₅₀ values comparing to the parent nucleoside **9.3**. In the L-alanine series, entries **9.6d-l**, different types of aliphatic **9.6d-f**, cyclic **9.6g-j** and aromatic esters **9.6k** were investigated. Amongst this family the most active compound was **9.6d** (α-naphthyl-L-alanine-*O*-propyl analogue) with EC₅₀ value of 0.0074 μM, showing more than 800-fold enhancement in the inhibition of the viral replication comparing to the parent nucleoside **9.3** (Table 9.7).

Not much difference in anti-HCV activity between L-alanine compounds bearing straight aliphatic, cyclic or aromatic esters was observed. All of these compounds exhibited the antiviral activity in ca. 10-20 nM range. Somewhat surprising result (EC₅₀ = 0.018 μM, Table 9.7) was obtained for compound **9.6j**. In the previous families of ProTides, the tetrahydropyranyl derivative was always less active than the corresponding cyclohexyl analogue (EC₅₀ = 0.17 μM vs. 0.03 μM for **5.12h** and **5.12c**, respectively, Chapter 5). As a factor responsible for that loss of activity we suggested relatively low ClogP value of the THP compound. In the case of **9.6j** introduction of the iodine atom in the C8-position resulted in the increase of the lipophilicity (ClogP = 1.1 for non-iodinated **5.12h** and 1.9 for **9.6j**) and most probably in enhanced cellular uptake and subsequently in the greater potency. The introduction of L-methionine, entries **9.6m-n**, provided compounds with submicromolar activity (EC₅₀ = 0.07 μM and EC₅₀ = 0.034 μM, respectively, Table 9.7), however no improvement over corresponding L-alanine derivatives was observed, confirming the superior role of L-alanine in the ProTide motif.

The variation of the aryl unit, replacement of α -naphthyl (**9.6a**, $EC_{50} = 0.02 \mu\text{M}$, Table 9.7) by phenyl (**9.6l**, $EC_{50} = 0.03 \mu\text{M}$, Table 9.7) did not result in noticeable difference in the replicon assay, showing that the 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine nucleoside analogue do not greatly distinguish between L-alanine esters or aromatic units. The replicon activities for eleven different L-alanine ProTides ranged from $0.01 \mu\text{M}$ to $0.04 \mu\text{M}$ (regardless the L-alanine ester or aromatic moiety used), only 2- to 4-fold, which is similar to the variability in the replicon assay.

Looking at diamidate series (Table 9.6 entry **9.9a**, Table 9.7 entries **9.9b-d**) it can be seen that the most active compounds were bearing L-alanine-*O*-benzyl ester (**9.9d**, $EC_{50} = 0.046 \mu\text{M}$, Table 9.7), followed by L-alanine-*O*-3,3-dimethyl-1-butyl ester (**9.9c**, $EC_{50} = 0.072 \mu\text{M}$, Table 9.7). Compounds **9.9a** and **9.9b**, were found to be equipotent ($EC_{50} = 0.13 \mu\text{M}$), and at the same time the least active across the whole family of synthesised prodrugs, although still more than 40-fold potent than the parent nucleoside **9.3**.

9.3.4 Enzymatic studies in the presence of carboxypeptidase Y.

To probe the mechanism of activation of newly synthesised prodrugs of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine carboxypeptidase assays were performed (Figure 9.4 and 9.5) according to the procedure described in Chapter 4, Section 4.6.

In the first instance carboxypeptidase Y assay was performed on α -naphthyl propylalanine analogue **9.6d** (Figure 9.4). In the blank experiments two peaks at $\delta_{\text{p}} = 3.80$ and 3.18 ppm corresponding to the two diastereoisomers of **9.6d** are present. During the first 7 min after enzyme addition, new peak at $\delta_{\text{p}} = 4.82 \text{ ppm}$ started to form and continued to be present for ca. 50 min. This intermediate corresponds to the compounds with the chemical structure **9.10**. Notably one of the diastereoisomers represented by the more downfield peak, was processed slightly faster. The half-life of **9.6d** was found to be 35 min. In comparison the half-life of **INX-189** was ca. 20 min.

The final product of the hydrolysis, represented by the single peak at $\delta_{\text{p}} = 7.37 \text{ ppm}$, corresponds to the aminoacyl intermediate represented by the chemical structure **9.11**. The full conversion of the parent ProTide was achieved within ca. 4 h.

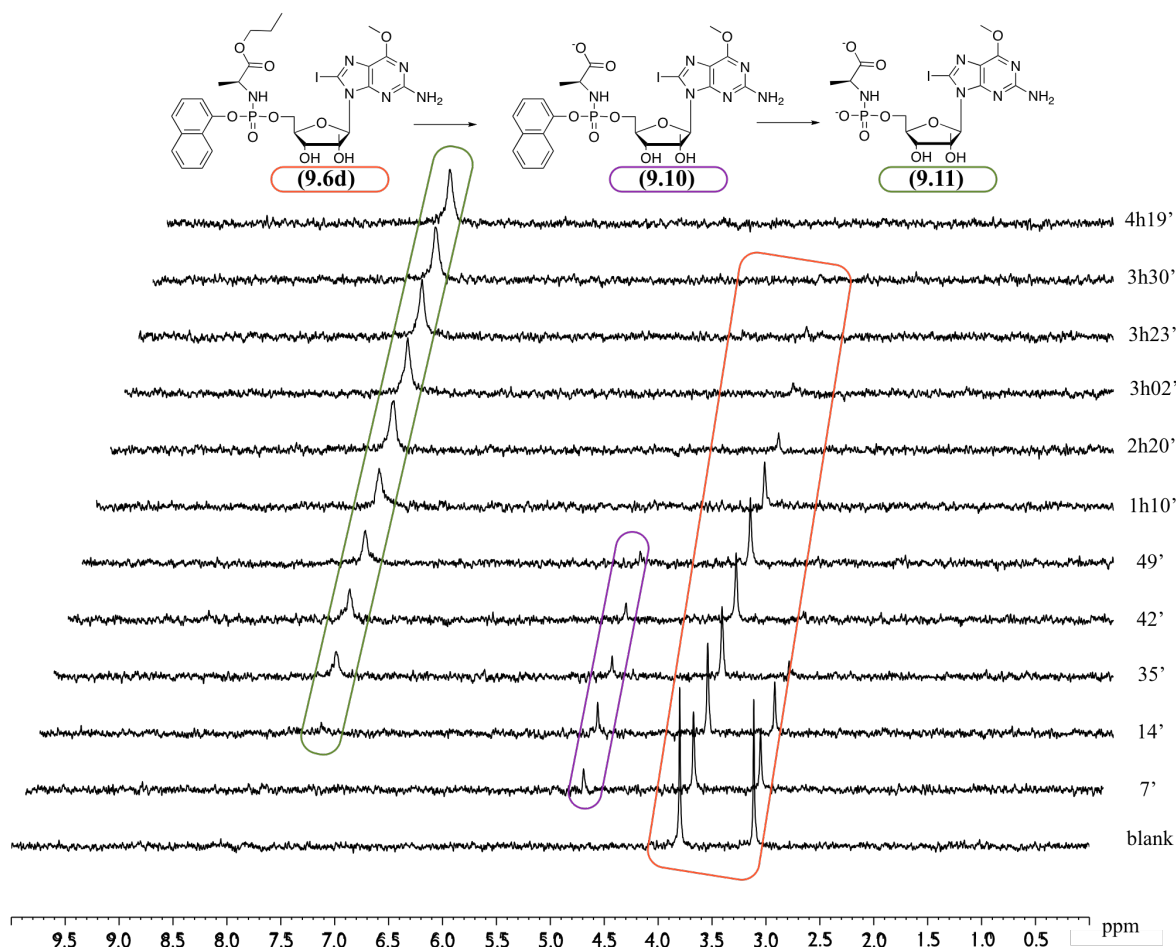


Figure 9.4. Carboxypeptidase Y mediated hydrolysis of **9.6d**, followed by ^{31}P NMR.

Efficient activation of **9.6d** is consistent with its exceptional replicon anti-HCV activity (Table 9.7).

Similarly, carboxypeptidase Y assay performed on L-alanine benzyl diamidate derivative **9.9d**, showed good conversion of the parent diamidate (Figure 9.5).

In the blank experiment **9.9d** has a ^{31}P NMR shift of 13.86 ppm (Figure 9.5). After 10 minutes of enzyme addition, a small downfield metabolite peak is observed at 14.28 ppm, which is consistent with cleavage of the benzyl ester moieties. The peak at 14.28 ppm builds up for approximately 50 min and after that time slowly diminishes with the concurrent formation of a new peak at 7.37 ppm. This intermediate starts to form within the first 17 min and grows in the magnitude over the course of the experiment. The peak at 7.37 ppm corresponds to the aminoacyl metabolite **9.11**. The half-life of **9.9d** was found to be less than 20 min.

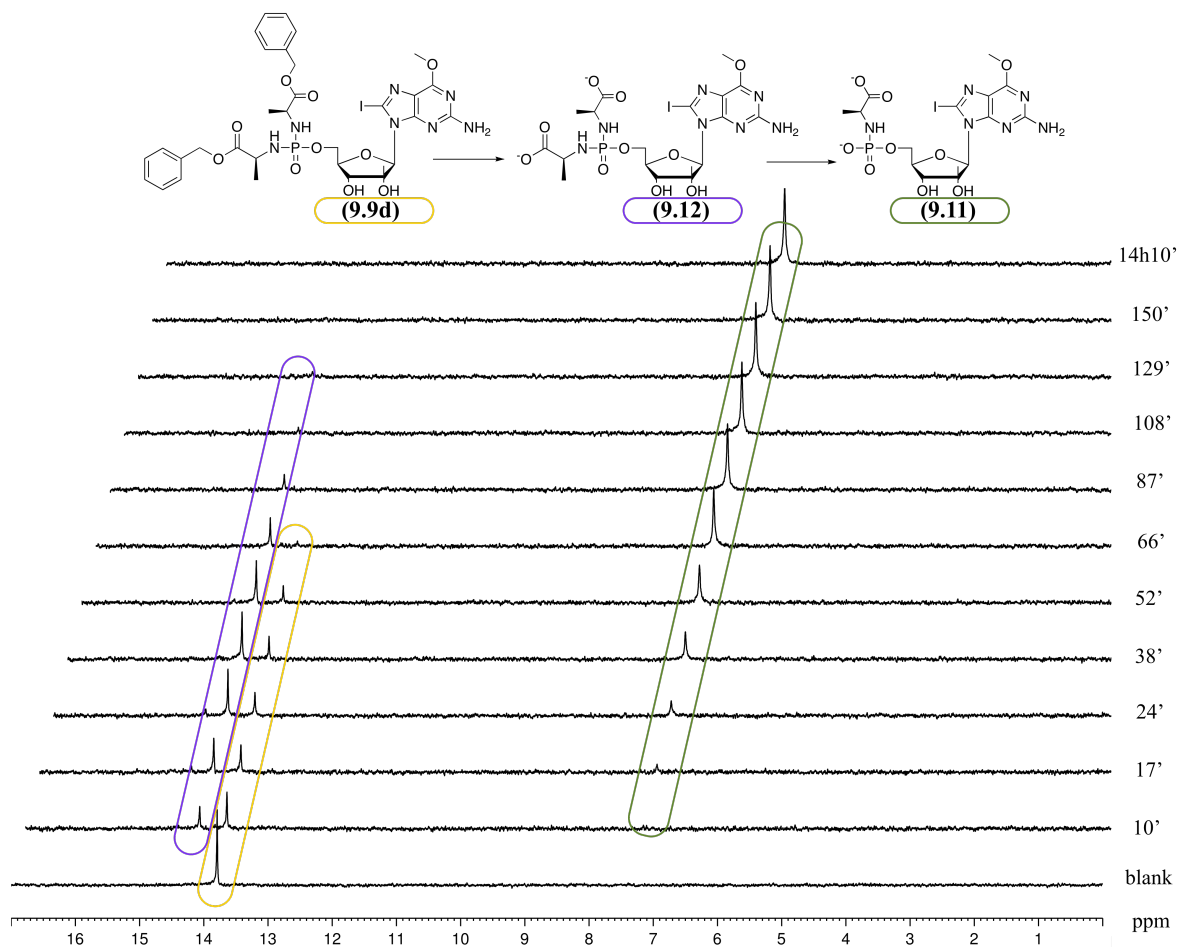


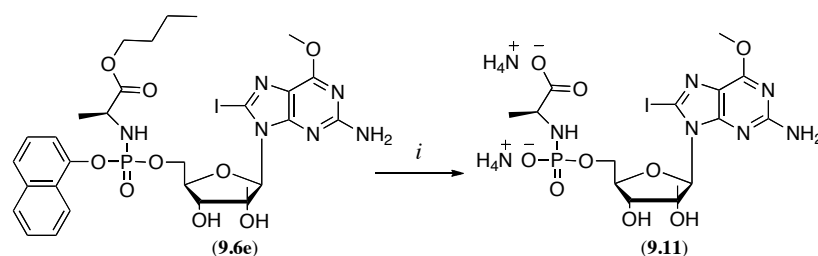
Figure 9.5. Carboxypeptidase Y mediated hydrolysis of **9.9d**, followed by ^{31}P NMR.

9.3.5 Synthesis of metabolic intermediate **9.11**.

In order to verify chemical structure of aminoacyl metabolite **9.11** formed during carboxypeptidase Y assay, synthesis of **9.11** was performed (Scheme 9.8). We were interested to see, whether the iodine atom is not being lost/metabolised during the initial steps of produg activation. To investigate that, UV spectra of **9.11** obtain during enzymatic and chemical hydrolysis and also non-iodinated analogue **5.21** from enzymatic assay were compared. The presence of the iodine in C8-position would result in noticeable shift of λ_{max} value comparing to the non-iodinated compound.

Synthesis of the diacid intermediate **9.11** involved hydrolysis of the previously prepared phosphoramidate **9.6e** (Scheme 9.8) in a 1:1 mixture of triethylamine and water. The reaction mixture was stirred at 40 °C for 6 days and after that time purified on silica gel,

using isopropanol : water : ammonia (8:1:1) as an eluent. Pure compound was obtained as diammonium salt.



Reagents and Conditions: i) Et₃N : H₂O (1:1), 40 °C, 6 days.

Scheme 9.8. Synthesis of 8-C-iodo-6-O-methyl-2'-C-methylguanosine aminoacyl intermediate **9.11**.

The structure of compound **9.11** was confirmed by NMR analysis and mass spectrometry.

With analytical marker **9.11** in hand, UV spectra comparison was performed (Figure 9.6).

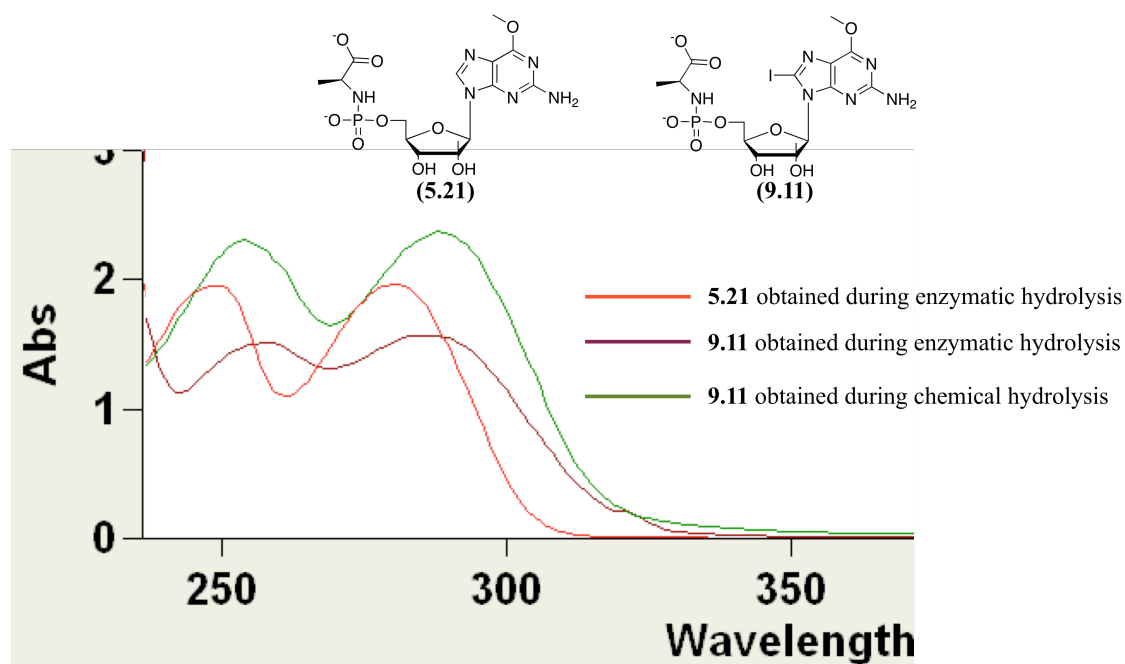


Figure 9.6. UV spectra comparison of metabolic intermediates **9.11** and **5.21**.

It is clear from Figure 9.6, that metabolic intermediate **9.11** obtained during enzymatic hydrolysis and one prepared synthetically share the same base structure, indicated by the same value of $\lambda_{\text{max}} = 288 \text{ nm}$ (red and brown curve). Non-iodinated analogue **5.21**

shows maximum absorbance at $\lambda_{\text{max}} = 280 \text{ nm}$. These data evidently show that in the presence of carboxypeptidase Y mediated hydrolysis of an ester unit the C8-iodo substituent stays intact.

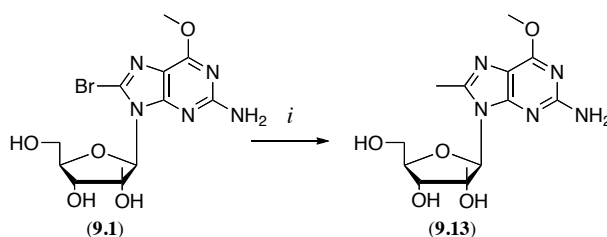
9.4 Synthesis of a second series of 8-modified nucleosides.

Encouraged by the results obtained for the C8-iodo nucleoside we decided to replace the C8-iodo atom by methyl-, thiomethyl-, methoxy- and phenyl- groups to mimic the iodine electronically and/or sterically and to investigate the influence on the antiviral potency.

9.4.1 Synthesis of 8-*C*-methyl-6-*O*-methyl-2'-*C*- β -methylguanosine.

Application of cross-coupling reactions in the synthesis of purines and nucleosides began in late 80's. At the beginning only rather scattered applications of reactions of halopurines with arylmagnesium halides, alkylcuprates and alkenylstannanes were reported.¹⁰ Introduction of various carbon chains onto the C-8 carbon atom of the naturally occurring purine nucleosides was mainly achieved via application of radical reactions or C-lithiation.¹¹ These methods however do not provide satisfactory regioselectivity and yields.

Although the cross-coupling of Grignard reagents with aryl halides proved to be very successful in synthetic organic chemistry, however the application to the 8-bromopurine was inefficient.¹² Hirota *et al.* reported the cross-coupling reaction of protected 8-bromoadenosine with trialkylaluminums in the presence of palladium catalyst in good yields.¹³ Thus, following the procedure, 8-*C*-methyl-6-*O*-methyl-2'-*C*-methylguanosine was synthesised in one-pot reaction from 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine (Scheme 9.9).



Reagents and Conditions: i) a) HMDS, $(\text{NH}_4)_2\text{SO}_4$, 1,4-dioxane, 3 h, reflux; then b) $\text{Al}(\text{Me}_3)_3$, $\text{Pd}(\text{PPh}_3)_4$, THF, 70°C ; b) NH_4Cl , MeOH, reflux, 3 h.

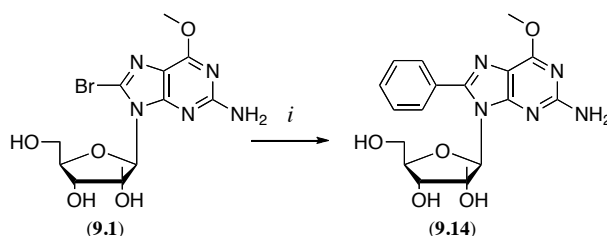
Scheme 9.9. Synthesis of 8-*C*-methyl-6-*O*-methyl-2'-*C*-methylguanosine **9.13**.

The conversion of the 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine was performed according to the slightly modified procedure: A mixture of 8-bromo nucleoside analogue was refluxed for 3 h in 1,4-dioxane, in the presence of excess of HMDS and a catalytic amount of ammonium sulfate, under an argon atmosphere. After solvents removal, the residue was dissolved in dry THF and tetrakis palladium triphenylphosphine was added, followed by the addition of trimethylaluminum solution. The reaction mixture was refluxed for 18 h. Removal of trimethylsilyl protecting groups was achieved in the presence of ammonium chloride in methanol. After column chromatography on silica gel, using CHCl₃ : MeOH (0 to 5%, gradient) as an eluent, pure compound was obtained as a yellowish solid in overall 72% yield.

9.4.2 Synthesis of 8-*C*-phenyl-6-*O*-methyl-2'-*C*-β-methylguanosine.

Although palladium catalysed cross-coupling reactions are widely used to obtain *C*-modified nucleosides, there are only few examples of palladium-catalysed syntheses of 8-arylpurine nucleosides. A Suzuki coupling of 8-halopurines and appropriate arylboronic acids was investigated. The typical Suzuki cross-coupling reaction is carried out under anhydrous conditions in nonpolar solvent with protected nucleoside to increase its lipophilicity. The necessary protection/deprotection adds additional two steps to the synthetic scheme and might cause decrease in overall reaction yield.

Western *et al.* reported efficient synthesis of 8-aryl-2'-deoxyguanosine in an aqueous solvent from unprotected halonucleoside in the presence of palladium catalyst and water-soluble phosphine ligand.¹⁴ It was decided to adopt this procedure in order to synthesise 8-*C*-phenyl-6-*O*-methyl-2'-*C*-methylguanosine (Scheme 9.10).



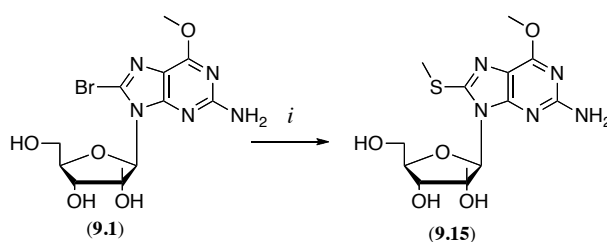
Reagents and Conditions: *i*) Pd(OAc)₂, TPPTS, Na₂CO₃, phenylboronic acid, H₂O : ACN (2:1), 80 °C, 4 h.

Scheme 9.10. Synthesis of 8-*C*-phenyl-6-*O*-methyl-2'-*C*-methylguanosine **9.14**.

The desired 8-aryl nucleoside analogue **9.14** was obtained in a single step reaction from unprotected 8-*C*-bromo-6-*O*-methyl-2'-*C*-methylguanosine **9.1**. Reaction was carried out in degassed water : acetonitrile mixture (2:1) in the presence of palladium acetate (Pd(OAc)₂) and 3,3',3''-phosphinidynetris(benzenesulfonic acid) trisodium salt (TPPTS) as a ligand under argon atmosphere, at 80 °C for 4 h. Degassing of the solvent system was found to be crucial for the good conversion of the starting material. After work-up and recrystallisation from water, pure **9.14** was obtained as a white fine powder, in very good 75% yield.

9.4.3 Synthesis of 8-*C*-thiomethyl-6-*O*-methyl-2'-*C*-β-methylguanosine.

Compounds bearing 8*C*-thiomethyl substituent was synthesised according to the procedure described for the 6-Cl displacement reactions described in detail in Chapter 5, Section 5.2.



Reagents and Conditions: i) NaSMe (15% in H₂O), DMF, rt, 2 h.

Scheme 9.11. Synthesis of 8-*C*-thiomethyl-6-*O*-methyl-2'-*C*-methylguanosine **9.15**.

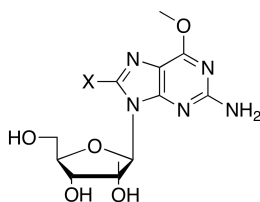
In the case of nucleoside analogue **9.15**, better results in terms of starting material conversion and ease of purification, were obtained when sodium methanethiolate was added portion wise over a period of 2 h.

After purification on silica gel, using CHCl₃/MeOH (0 to 5% in gradient) as an eluent, pure **9.15** was obtained as a yellowish solid in quantitative yield.

9.4.4 Biological evaluation of a second series of 8-modified 6-*O*-methyl-2'-*C*- β -methylguanosine nucleoside analogues.

All newly synthesised nucleoside analogues **9.13-9.15** were investigated for their ability to inhibit HCV replication in *in vitro* cell-based assay by Inhibitex Inc.

The corresponding data are reported in Table 9.8.



| Cpd | X | EC ₅₀ [μ M] | CC ₅₀ [μ M] | ClogP | Yield % |
|-------------|-------------------------------|-----------------------------|-----------------------------|-------|---------|
| 5.4 | H | 4.6 | >100 | -0.6 | - |
| 9.3 | I | 6.0 | >100 | 0.2 | 51 |
| 9.13 | CH ₃ | >100 | >100 | -0.4 | 72 |
| 9.14 | C ₆ H ₅ | 58 | >100 | 1.4 | 75 |
| 9.15 | SCH ₃ | >100 | >100 | 0.5 | 98 |

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%;
CC₅₀: 50% cytotoxic concentration.

Table 9.8. Summary of the anti-HCV activity of a second series of 8-*C*-modified-6OMe2'-CMeG nucleoside analogues.

As can be seen in Table 9.8, all C8-modified nucleoside analogues possess increased ClogP values comparing to the 6-*O*-methyl-2'-*C*-methylguanosine with no modification at C8-position. The lowest ClogP value was reported for **9.13** C8-methyl analogue (ClogP = -0.4) and the highest for **9.14** bearing C8-phenyl alteration (ClogP = 1.4).

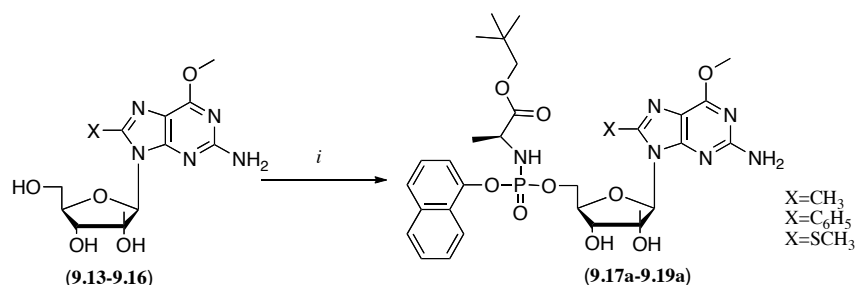
From the replicon data it can be noticed that none of the newly synthesised analogues of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine exhibit significant anti-HCV activity. Compounds bearing simple methyl- **9.13** and thiomethyl- **9.15** substituents at C8-position were found to be completely inactive in replicon assay. In parallel experiments analogue with 8-*C*-phenyl modification was exhibiting low anti-HCV activity, with EC₅₀ values in a range of 50 μ M.

All synthesised nucleoside analogues did not display any toxicity up to 100 μ M in replicon assay.

It was decided to apply ProTide technology to the second series of 8C-modified nucleoside analogues in order to investigate potential improvement of antiviral activity of these compounds.

9.5 SAR of a second series of 8-modified 6-*O*-methyl-2'-*C*- β -methylguanosine ProTides.

A small series of 5'-phosphoramidates (**9.17a-9.19a**) was prepared using *t*BuMgCl-mediated coupling conditions, in moderate yield 7-23% (Scheme 9.12, Table 9.9). To facilitate the comparison, all compounds were prepared as α -naphthyl-L-alanine-*O*-2,2-dimethylpropyl derivatives.



Reagents and Conditions: i) Naph-L-alanine-*O*-2,2-dimethylpropyl phosphorochloridate, *t*BuMgCl, dry THF, rt, overnight.

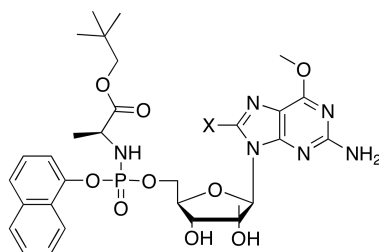
Scheme 9.11. General procedure for the synthesis of a second series of 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine ProTides using *t*BuMgCl.

After column chromatography, pure compounds **9.17a-9.19a** were obtained as equimolar mixtures of phosphate diastereoisomers as evidenced by ^{31}P NMR and HPLC.

All newly synthesised compounds were assayed for their ability to inhibit HCV RNA replication in subgenomic replicon cells by Inhibitex Inc. The potency and toxicity of these compounds are summarised in Table 9.9. The **INX-189** and **9.6a**- 8C-iodo analogue were evaluated alongside and were used as a references.

Considering the lipophilicity, all synthesised analogues have ClogP values in a range of 3.5-5.5 (Table 9.9), which is considered as an optimal for the passive cellular uptake. As noted in Table 9.9, phosphoramidate **9.17a** (8C-methyl) was poorly active against HCV replicon cells. Its EC_{50} value was found to be greater than 30 μM ; at the same time the compound did not display any cellular toxicity up to 100 μM concentration. The C8-thiomethyl derivative **9.19a** exhibited improved potency comparing to the parent nucleoside

9.15 ($EC_{50} = 13 \mu\text{M}$ vs. $EC_{50} > 100 \mu\text{M}$, respectively), however this activity was more than a 1000-fold lower than the one of clinical candidate **INX-189** ($EC_{50} = 0.01 \mu\text{M}$). Additionally some toxicity ($CC_{50} = 39 \mu\text{M}$) related to **9.19a** was observed.



| Cpd | X | EC_{50} [μM] | CC_{50} [μM] | ClogP | Yield % |
|----------------|-------------------------------|-----------------------------|-----------------------------|-------|---------|
| INX-189 | H | 0.01 | 7 | 3.3 | 27 |
| 9.6a | I | 0.02 | 14 | 4.1 | 27 |
| 9.17a | CH ₃ | 37 | >100 | 3.5 | 14 |
| 9.18a | C ₆ H ₅ | 3.6 | 22 | 5.4 | 7 |
| 9.19a | SCH ₃ | 13 | 39 | 4.5 | 23 |

EC_{50} : 50% effective concentration or compound concentration required to inhibit HCV replication by 50%;
 CC_{50} : 50% cytotoxic concentration.

Table 9.9. Summary of the anti-HCV activity of a second series of 8-C-modified-6OMe2'CMeG ProTides, their calculated lipophilicity values and isolated yields.

The most interesting compound across the family was 8-C-phenyl derivative **9.18a**, exhibiting anti-HCV activity in low micromolar range ($EC_{50} = 3.6 \mu\text{M}$). This is the only example (except the 8-C-iodo series) of 8-modified ProTide displaying inhibitory activity below $5 \mu\text{M}$ concentration. This particular compound is a proof of concept example where application of ProTide technology allowing to convert a very poorly active nucleoside **9.14** ($EC_{50} = 58 \mu\text{M}$) into compound with low micromolar activity.

Although the activity of **9.18a** is greatly decreased comparing to the activity of **INX-189**, the family of novel 8-C-phenyl analogues can become a starting point in the design and synthesis of a new class of inhibitors with improved potency and lower toxicity.

9.6 Understanding the 8-*C*-iodo phenomena. Analysis of hepatocyte metabolites of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine ProTides.

Several novel 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine nucleoside analogues were designed and synthesised, and ProTide technology was applied to each one. Across seven different modifications only 8-*C*-iodo derivatives exhibited potency comparable to that of clinical candidate **INX-189**. From all experiments performed it was clear that the presence of iodine is essential for the activity.

In order to understand the phenomenon of the iodine substituent, the following metabolic pathway was suggested (Figure 9.7).

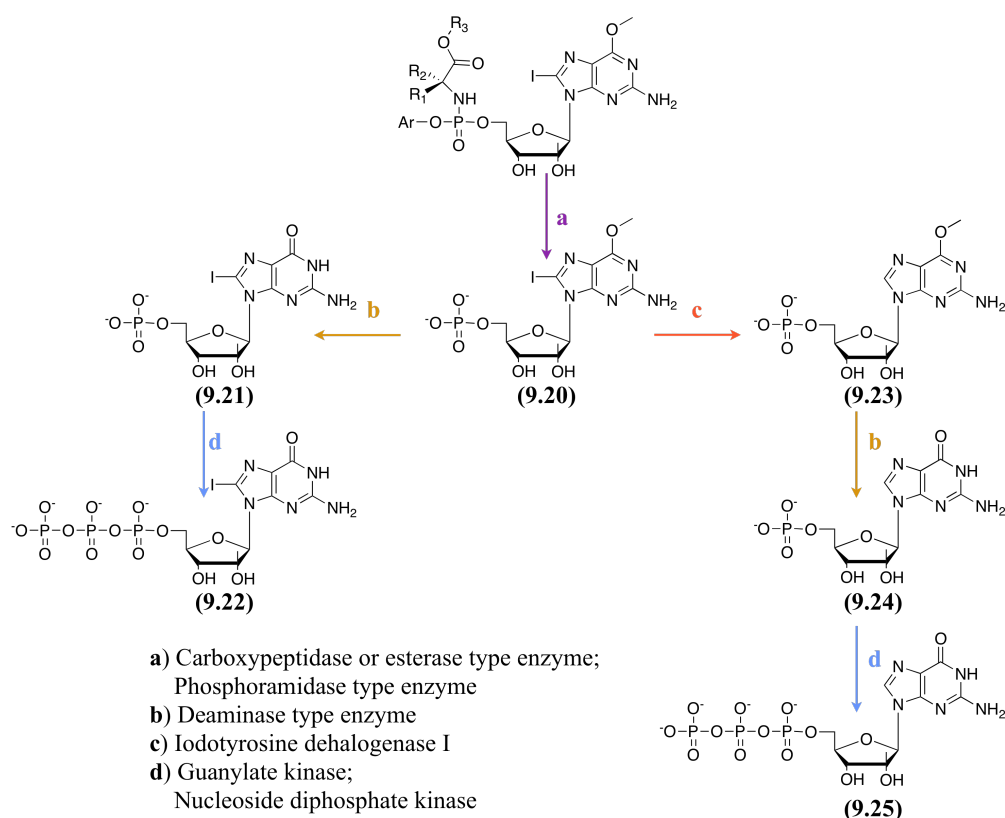


Figure 9.7. Suggested metabolic pathway of 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine ProTides.

The activation of the 8-*C*-iodo ProTide could involve 'traditional' conversion to the 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine 5'-monophosphate **9.20**. At this point it is not clear whether the **9.20** could serve as a substrate for adenylate deaminase or any other deaminating enzyme to give 8-*C*-iodo-2'-*C*-methylguanosine-5'-monophosphate **9.21** which

could undergo further phosphorylation to the active 8-*C*-iodo-2'-*C*-methylguanosine-5'-triphosphate **9.22**. An experiment performed in the presence of ADA (Section 9.2.5) shown that 8-*C*-iodo-6-*O*-methyl-2'-*C*-methylguanosine **9.3** (parent nucleoside) is not susceptible substrate for the enzyme. Taking in consideration substrate specificity of adenylylate deaminase it is possible that this enzyme could not be responsible for the hydrolysis of C6-substituent. Also due to the fact that there are many different type of deaminases able to deaminate adenosine monophosphate, this particular metabolic pathway cannot be excluded. On the other hand it was considered that the monophosphate **9.20** could undergo a deiodination reaction. We wondered if it may be mediated by iodotyrosine dehalogenase I (iodotyrosine deiodinase, IYD, EC 1.22.1.1). This enzyme is responsible for iodide salvage, catalysing deiodination of mono- and diiodotyrosine- halogenated by-products of thyroid hormone production. It has been reported that deiodination occurs predominantly in the liver microsomes and is mediated by NADPH. Molecular modelling studies¹⁵ (data not shown) suggest good fitting of **9.20** in the active site of IYD. After deiodination **9.23** could undergo ADA-mediated hydrolysis to form **9.24** and consequently after phosphorylation to the active 2'-*C*-methylguanosine-5'-triphosphate species **9.25**. The deiodination step could explain the specific activity of 8-*C*-iodo nucleoside and related prodrugs comparing to any other C8-modified analogues.

To probe the suggested metabolic pathway of 8-*C*-iodo prodrugs, the analysis of metabolites produced during incubation of 8-iodinated ProTides in the presence of primary human hepatocytes was performed. Experiments were conducted by Inhibitex Inc.

ProTides **9.6a** and **INX-189** were incubated in primary human hepatocytes at an arbitrary concentration of 10 μ M over a period of 24 h. ProTide uptake was measured in cell supernatant (Figure 9.8) and metabolic products were measured intracellularly at 0.5 h, 1h, 2 h, 4 h, 6 h, 8 h and 24 h time points (Figure 9.9).

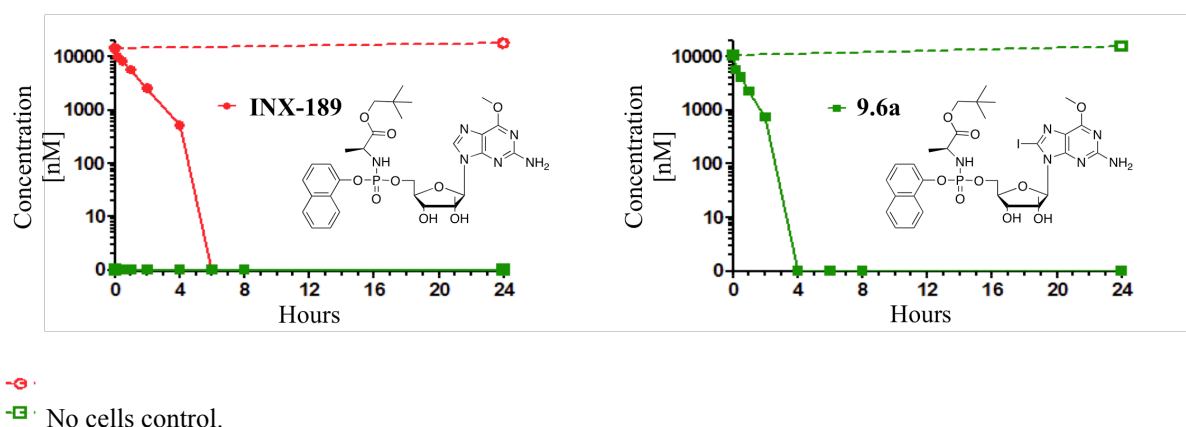


Figure 9.8. Cellular uptake of **INX-189** and **9.6a** during incubation with primary human hepatocytes.

Data reported in Figure 9.8 clearly show rapid cellular uptake of both ProTides. In the case of **INX-189** no traces of prodrug in cell supernate were detected after 6 h of incubation. In comparison **9.6a** was found to be delivered faster inside the cell, within ca. 4 h. This difference can arise from improved lipophilicity of **9.6a** compared to **INX-189** (ClogP = 4.1 vs. 3.3, respectively).

In the next instance intracellular levels of metabolite formed during incubation with primary hepatocytes were measured (Figure 9.9).

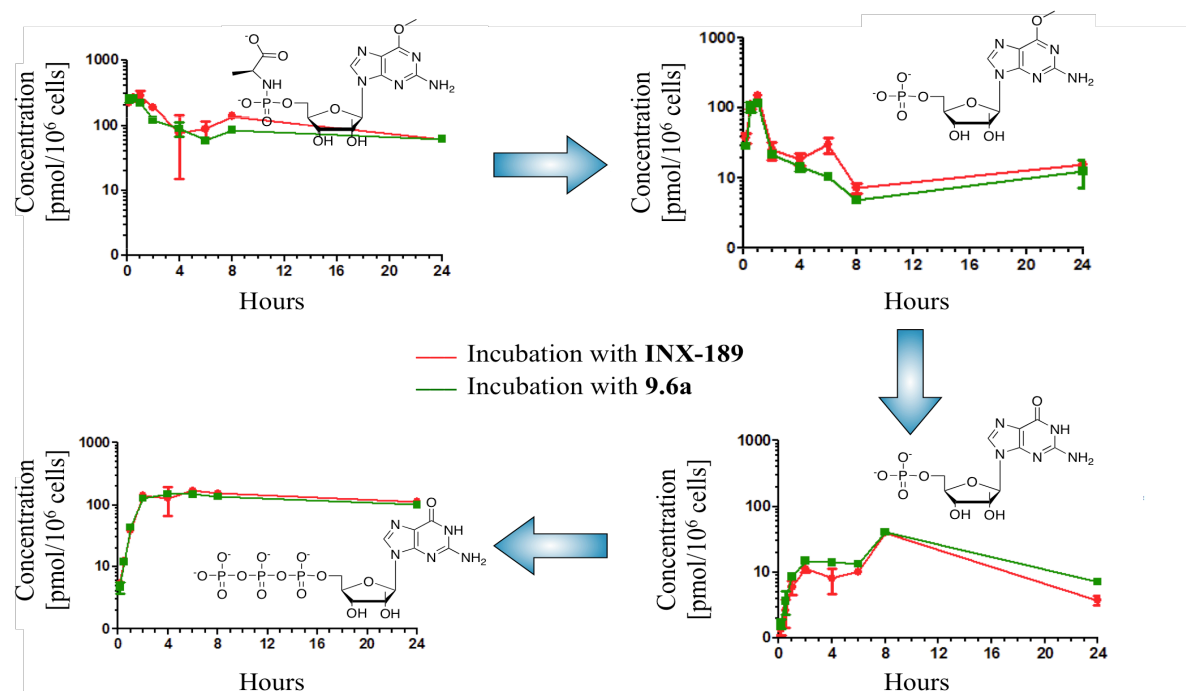


Figure 9.9. Intracellular metabolites produced from **9.6a** and **INX-189** during incubation in the presence of primary human hepatocytes.

Results obtained from the analysis of hepatocyte intracellular fractions (Figure 9.9) clearly show, that there has been no difference between the levels of individual metabolites including the active 2'CMeG 5'-triphosphate produced from the incubation of either **9.6a** or **INX-189** with primary human hepatocytes. These data are consistent with biological activity observed for these compounds. Furthermore no traces of 8-*C*-iodo analogues of any of the metabolites were detected.

These results are somewhat surprising, as the iodo substituent did not display any profound instability, however it is obvious that the 8-*C*-iodo comes off very quickly (once in the presence of biological media), apparently before the cleavage of the phosphoramidate amino acid ester.

Data reported in Figure 9.9 explain the high replicon activity of 8-*C*-iodo derivatives comparing to any other 8-*C*-modified analogues and make clear that the introduced 8-*C*-iodo substituent act as a promoiety.

9.7 Conclusions.

To summarise, a series of novel 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine nucleoside analogues and their aryloxyphosphoramidate, and diamidate prodrugs has been synthesised. During biological evaluation it was found, that only analogues bearing 8-*C*-iodo substituent displayed anti-HCV inhibitory activity. Structure activity relationships were extensively studied, including modification in the ester, aryl and amino acid moieties. A combination of various esters and amino acids resulted in very potent compounds displaying nanomolar activity, suitable for further development.

Evaluation of a second family of 8-*C*-modified nucleosides and ProTides designed to mimic the 8-*C*-iodo sterically and/or electronically showed that only 8-*C*-phenyl derivative exhibit some potential, however these compounds were ca. 200-fold less potent than the corresponding iodo analogues and ca. 400-times less active than the clinical candidate **INX-189**.

To understand the phenomenon of the 8-*C*-iodo substituent, metabolite analyses of intermediates formed during incubation of appropriate ProTides with primary human hepatocytes were conducted. Obtained data revealed that the 8-*C*-iodo substituent act as a promoiety with eventual liberation of 2'CMeGTP, as with the clinical candidate **INX-189**.

References:

1. Robak T, Lech-Maranda E, Korycka A, Robak E. Purine nucleoside analogues as immunosuppressive and antineoplastic agents: mechanism of action and clinical activity. *Curr Med Chem* **2006**, 13, 3165-3189.
2. Bennett LL Jr, Chankg CH, Allan PW, Adamson DJ, Rose LM, Brockman RW, Secrist JA III, Shortnacy A, Montgomery JA. Metaboslim and metabolic effects of halopurine nucleosides in tumor cells in culture. *Nucleosides and Nucleotides* **1985**, 4, 107-116.
3. Goodman MG. Role of salvage and phosphorylation in the immunostimulatory activity of C8-substituted guanine ribonucleosides. *J Immunol* **1988**, 141 (7), 2394-2399.
4. Clark AS, Stevens MF, Sansom CE, Schwalbe CH. Anti-tumour imidazotetrazines. Part XXI. Mitozolomide and temozolomide: probes for the major groove of DNA. *Anticancer Drug Des* **1990**, 5, 63-68.
5. Gannett PM, Sura TP. An improved synthesis of 8-bromo-2'-deoxyguanosine. *Synth Commun* **1993**, 11, 1611-1615.
6. Ryu EK, Kim JN. The oxidative chlorination of pyrimidine and purine bases, and nucleosides using acyl chloride-methylformamide-*m*-chlorobenzoic acid system. *Nucleosides and Nucleotides* **1989**, 8(1), 43-48.
7. Hamm ML, Rajguru S, Downs AM, Cholera R. Base pair stability of 8-chloro- and 8-iodo-2'-deoxyguanosine opposite 2'-deoxycytidine: Implications regarding the bioactivity of 8-oxo-2'-deoxyguanosine. *J Am Chem Soc* **2005**, 127, 12220-12221.
8. Lipkin D, Howard FB, Nowotny D, Sano M. The iodination of nucleosides and nucleotides. *J Biol Chem* **1963**, 238, 2249-2251.
9. Balci M. Basic ^1H - and ^{13}C -NMR spectroscopy. Elsevier B.V. **2005**.
10. Cesnek M, Hocek M, Holy A. Synthesis of acyclic nucleotide analogues derived from 2-amino-6-C-substituted purines via cross-coupling reactions of 2-amino-9-[2-(diisopropoxyphosphorylmethoxy)-ethyl]-6-halopurines with diverse organometallic reagents. *Collect Czech Chem Commun* **2000**, 65, 1357-1373.
11. Tu C, Keane C, Eaton BE. Palladium catalysis in the synthesis of 8-position modified adenosine, 2'-deoxyadenosine and guanosine. *Nucleosides and Nucleotides* **1995**, 14(8), 1631-1638.

12. Cong-Dahn N, Beaucourt JP, Pichat L. Modification de la position 8 des purines nucleosides et de l'adenosine monophosphate cyclique-3',5'. Reactions de couplage catalytique des organomagnesium avec les bromo-8 purines ribosides et bromo-8 adenosine monophosphate cyclique-3',5' silyles en presence de dichloro-bis-triphenylphosphine palladium. *Tetrahedron Lett* **1979**, 34, 359-3162.
13. Hirota K, Kitade Y, Kanbe Y, Maki Y. Convenient method for the synthesis of C-alkylated purine nucleosides: Palladium-catalyzed cross-coupling reaction of halogenopurine nucleosides with trialkylaluminums. *J Org Chem* **1992**, 57, 5268-5270.
14. Western EC, Daft JR, Johnson EM II, Gannett PM, Shaughnessy KH. Efficient one-step Suzuki arylation of unprotected halonucleosides, using water-soluble palladium catalyst. *J Org Chem* **2003**, 68, 6767-6774.
15. Molecular modelling studies were performed by dr. A. Brancale at School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff.

CHAPTER TEN

New family: C-2 modifications.

10.1 2-C-modified-2'-C- β -methylguanosine – the rational behind the design.

It has been reported in the literature, that the 2C-N atom of guanine is susceptible to modifications by various potential carcinogens including formaldehyde, acetaldehyde and oxidation products of structurally distinctive aromatic hydrocarbons. Some of these 2C-N adducts were found to exhibit mutagenic properties by generating G to T transversion. The activity of C2-N substituted nucleoside analogues is based partly on their ability to mimic the base-pairing capacity of guanosine 5'-triphosphate. Furthermore it has been suggested, that the bulky group on 2C-N atom of guanine can cause strong interference with polymerisation reaction, leading to misincorporation and blockage of replicative DNA polymerases, most probably at or before the phosphodiester bond formation.¹⁻⁴

Several different substitutions in the C2-position of the purine base were investigated and docking studies using HCV polymerase model were performed by Brancale and Zonta.^{5a-b} Molecular modelling studies confirmed important role of the C2-nitrogen in hydrogen bond formation during base pairing. Consequently modifications which do not affect hydrogen bonding capabilities of the C2-nitrogen were well tolerated (even bearing bulky substituents) in the active site of the RdRp.

We decided to synthesise various C2-modified analogues of 2'-C- β -methylguanosine and investigate their influence on HCV replication.

10.2 Synthesis of 2C-modified derivatives – 2C-benzylamine analogue.

In the first instance we have focused our attention on modifications that would preserve the ability of the C2-group to form hydrogen bonding during base pairing. Molecular modelling studies suggested 2-C-benzylamine-2'-C- β -methylguanosine as an analogue that would fit the best in the active site of the RdRp (Figure 10.1).^{5a}

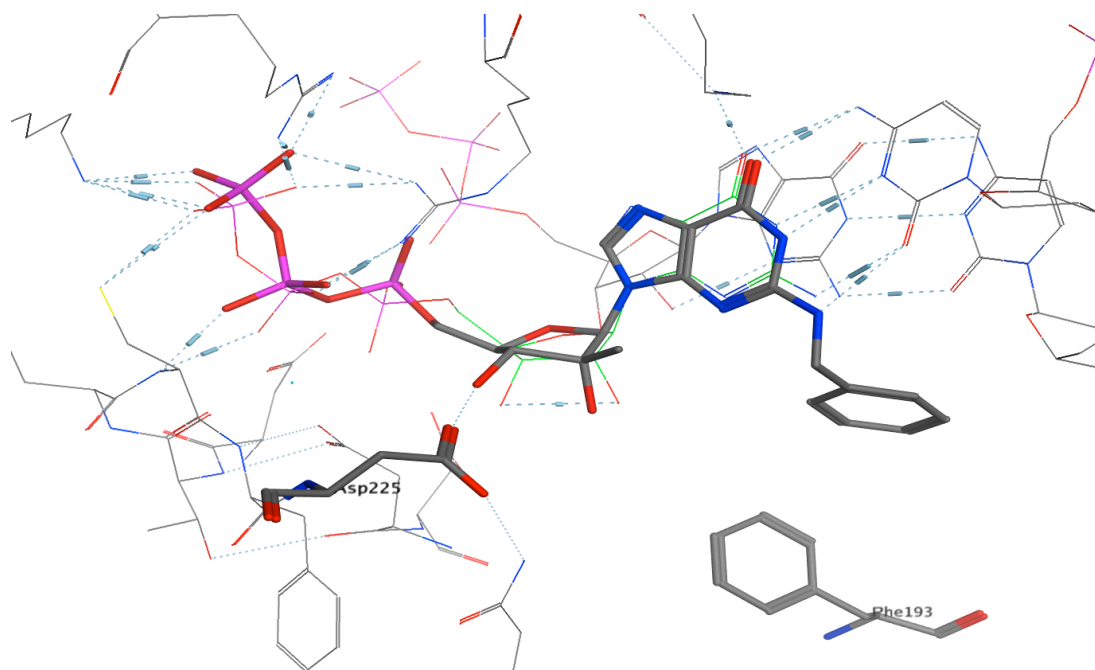
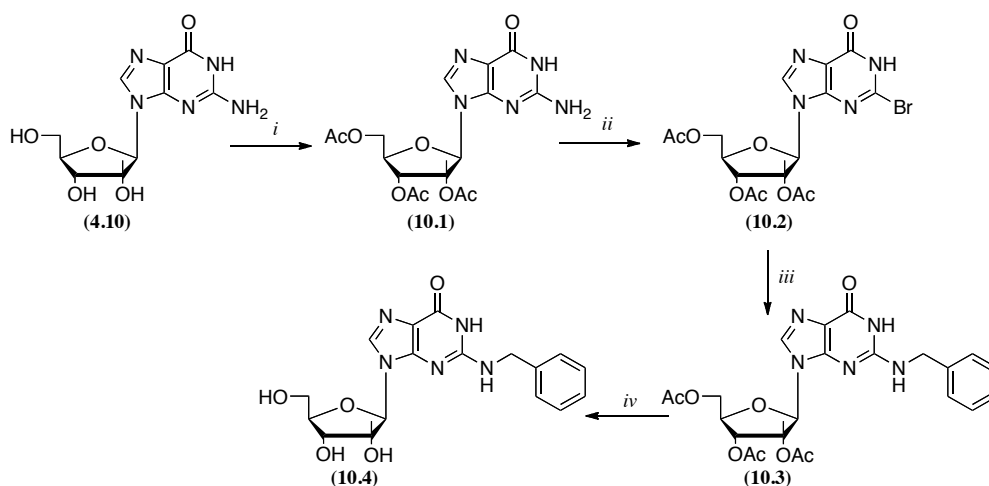


Figure 10.1. Docking results of the 2-C-benzyl-2'-C-methylguanosine in the active site of RdRp.^{5a}

10.2.1 Synthesis of 2-C-benzylamine-2'-C-β-methylguanosine via diazotization-halogenation reaction.

One of the reported routes towards 2-C-modified purine analogues involves diazotization/bromo dediazonation reaction followed by nucleophilic displacement of the halogen atom. It was decided to adopt the procedure described by Trivedi⁶ in order to synthesise 2-C-benzylamine-2'-C-β-methylguanosine (Scheme 10.1).



Reagents and Condition: i) Et_3N , DMAP, Ac_2O , ACN, rt, 4 h; ii) *i*-pentyl nitrite, CHBr_3 , 95 °C, 3 h; iii) PhCH_2NH_2 , EtOH, reflux, 16 h; iv) NH_3/MeOH , 0 C to rt, 16 h.

Scheme 10.1. Synthesis of 2-C-benzylamine-2'-C-methylguanosine via diazotization-halogenation reaction.

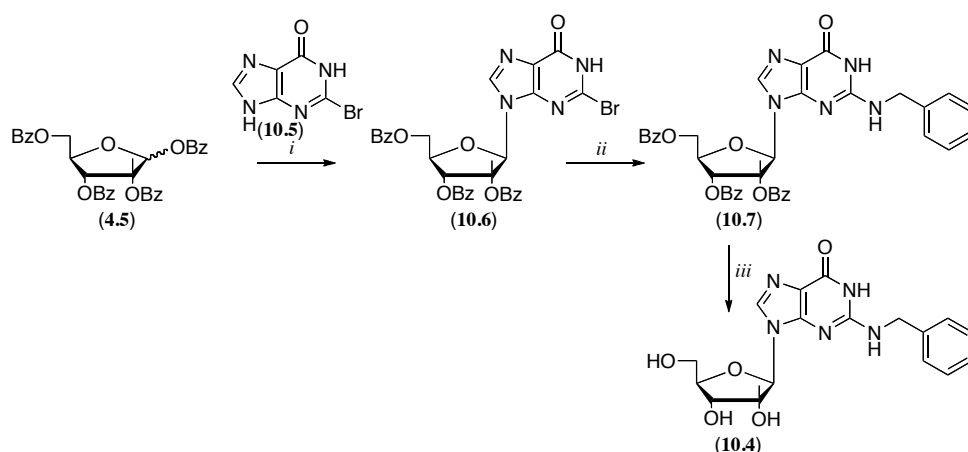
In the first step, 2'-C-methylguanosine **4.10** was fully protected with acetyl groups in 90% yield. Halogenation of 2'-C-methylguanosine 2',3',5'-triacetate **10.1** was performed in bromoform in the presence of isopentyl nitrite. After column chromatography pure compound **10.2** was obtained as a yellowish solid in 35% yield. The nucleophilic displacement with benzylamine in refluxing ethanol provided **10.3** in 33% yield. The final deprotection of acetyl protecting groups was accomplished using methanolic ammonia. After purification on silica gel using CHCl₃ : MeOH (8:2) as eluent, pure 2-C-benzylamine-2'-C-methylguanosine (**10.4**) was obtained in 74% yield.

As the overall yield of the reaction was lower than 8%, we decided to investigate different synthetic pathways in order to obtain **10.4** in more satisfactory yield.

10.2.2 Synthesis of 2-C-benzylamine-2'-C-β-methylguanosine via convergent method.

In order to obtain **10.4** in better yield, Vorbruggen coupling reaction of 1,2,3,5-tetra-*O*-benzoyl-2-C-methyl-D-ribofuranose (**4.5**) and commercially available 2-bromohypoxanthine (**10.5**) was investigated.

The coupling reaction was carried out in dry acetonitrile in the presence of DBU and TMS-triflate at 65 °C for 6-8 h (Scheme 10.2). After column chromatography, pure **10.6** was isolated as a yellowish solid in 78% yield. The introduction of benzylamine functionality was carried out using conditions described in the previous section and resulted in formation of **10.7** in 43% yield.



Reagents and Condition: i) DBU, TMSOTf, dry ACN, 65 °C, 6-8 h; ii) PhCH₂NH₂, EtOH, reflux, 16 h; iii) NH₃/MeOH, 0 °C to rt, 16 h.

Scheme 10.2. Synthesis of 2-C-benzylamine-2'-C-methylguanosine using convergent approach.

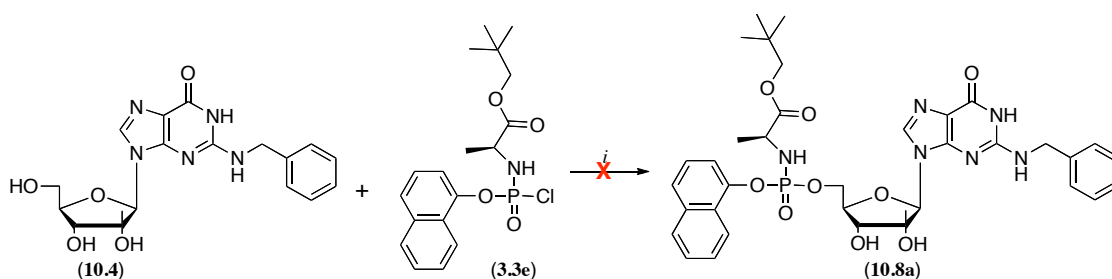
Subsequent deprotection step in methanolic ammonia followed by column chromatography, provided pure **10.4** in good yield (73%).

The new synthetic route resulted not only in improved overall yield (25% vs. 8%) but also involved fewer steps and reaction mixtures were easier to purify.

10.2.2.1 Synthesis of ProTides of 2-C-benzylamine analogues – first attempt.

With **10.4** in hand, direct coupling of nucleoside analogue and α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate was performed. In the first attempt, the general *t*BuMgCl mediated procedure (used for the synthesis of 6-*O*-methyl-2'-*C*-methylguanosine) was applied (Scheme 10.3, Table 10.1, Entry 1). During 16 h course of the reaction, no product formation was observed as evidenced by TLC analysis. After column chromatography only starting material was recovered.

After failure of the first attempt two additional reactions were set up. Firstly, reaction was carried out in elevated temperature at 35 °C (Table 10.1, Entry 2) and secondly 5 mol equivalents of phosphorochloridate were used (Table 10.1, Entry 3). As can be seen in Table 10.1. none of the described attempts resulted in product formation. Alteration of the reaction solvent was investigated in order to enhance solubility of the starting material **10.4**. The reaction was carried out in the mixture of THF and pyridine, and in the presence of NMI (Table 10.1, Entry 4) as an activator. The NMI-mediated coupling was applied instead of the Grignard reagent, as *t*BuMgCl was found to be not compatible with pyridine when used as a solvent.



Reagents and Condition: For reagents and conditions refer to Table 10.1.

Scheme 10.3. Attempted synthesis of 2-*C*-benzylamine-2'-*C*-methylguanosine ProTide.

| Entry | 10.4 | 3.3e | Solvent | Reagents | Conditions | yield % |
|-------|----------|----------|--------------|--------------------------|-------------|---------|
| 1 | 1 mol eq | 2 mol eq | THF | <i>t</i> BuMgCl 2 mol eq | rt, 16 h | - |
| 2 | 1 mol eq | 2 mol eq | THF | <i>t</i> BuMgCl 2 mol eq | 35 °C, 16 h | - |
| 3 | 1 mol eq | 5 mol eq | THF | <i>t</i> BuMgCl 5 mol eq | 35 °C, 16 h | - |
| 4 | 1 mol eq | 3 mol eq | THF/Pyridine | NMI 5 mol eq | rt, 16 h | - |
| 5 | 1 mol eq | 3 mol eq | Pyridine | NMI 5 mol eq | rt, 16 h | - |
| 6 | 1 mol eq | 3 mol eq | Pyridine | NMI 5 mol eq | 35 °C, 16 h | - |

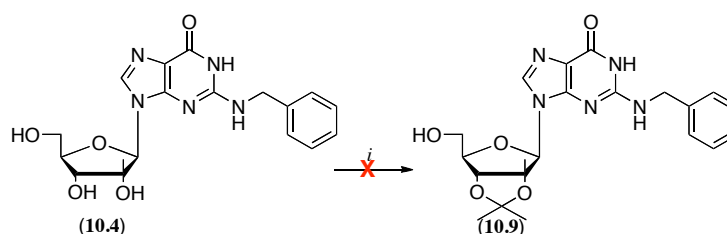
Table 10.1. Reagents and conditions of Scheme 10.3.

The reaction was carried out at ambient temperature over 16 h. During that time no product formation was observed. It was decided to use pyridine as a sole solvent for the reaction (Table 10.1, Entries 5 and 6), however no improvement either at ambient or elevated (35 °C) temperature was observed.

Due to the low solubility of 2-*C*-benzylamine-2'-*C*-methylguanosine in THF and pyridine, it was decided to investigate a nucleoside protection strategy.

10.2.2.2 2',3'-protection of 2-*C*-benzylamine-2'-*C*-methylguanosine.

To overcome solubility problems and to enhance 5'-phosphorylation, 2',3'-diol protection was investigated. As the method used for the preparation of 2',3'-isopropylidene-2'-*C*-methylguanosine was very successful (Chapter 4, Section 4.3), it was decided to apply the same reaction conditions to 2-*C*-benzylamine-2'-*C*-methylguanosine (Scheme 10.4, Table 10.2, Entry 1).



Reagents and Condition: For reagents and conditions refer to Table 10.2.

Scheme 10.4. Attempted synthesis of 2',3'-isopropylidene protected 2-*C*-benzylamine-2'-*C*-methylguanosine.

| Entry | Solvent | Reagents | Conditions | yield % |
|-------|-------------------------------|-------------------|------------|---------|
| 1 | Acetone | HClO ₄ | rt, 16 h | - |
| 2 | Acetone | HClO ₄ | 35 °C, 3 h | - |
| 3 | Acetone | <i>p</i> TSA | rt, 16 h | - |
| 4 | Acetone, 2,2-dimethoxypropane | <i>p</i> TSA | rt, 16 h | - |
| 5 | Acetone, 2,2-dimethoxypropane | <i>p</i> TSA | 35 °C, 1 h | - |
| 6 | DMF, 2,2-dimethoxypropane | <i>p</i> TSA | rt, 16 h | - |

Table 10.2. Reagents and conditions of Scheme 10.4.

As can be seen in Table 10.2, reaction carried out in acetone and in the presence of catalytic amount of perchloric acid, did not result in product formation. The TLC analysis of the reaction mixture indicated that mostly starting material **10.4** was present plus some decomposition products. Reduction of the reaction time with elevated temperature (Table 10.2, Entry 2) gave mainly decomposition (base line product) of the starting material **10.4**. The replacement of the acid catalyst by *p*TSA (Table 10.2, Entry 3) gave similar results as the first trial. The two subsequent reaction attempts (Table 10.2, Entry 4 and 5) carried out in the presence of acetone and 2,2-dimethoxypropane and catalytic amounts of *p*TSA, either at ambient temperature or at 35 °C, did not result in product formation. Change of the solvent from acetone to DMF also did not provide the desired product.

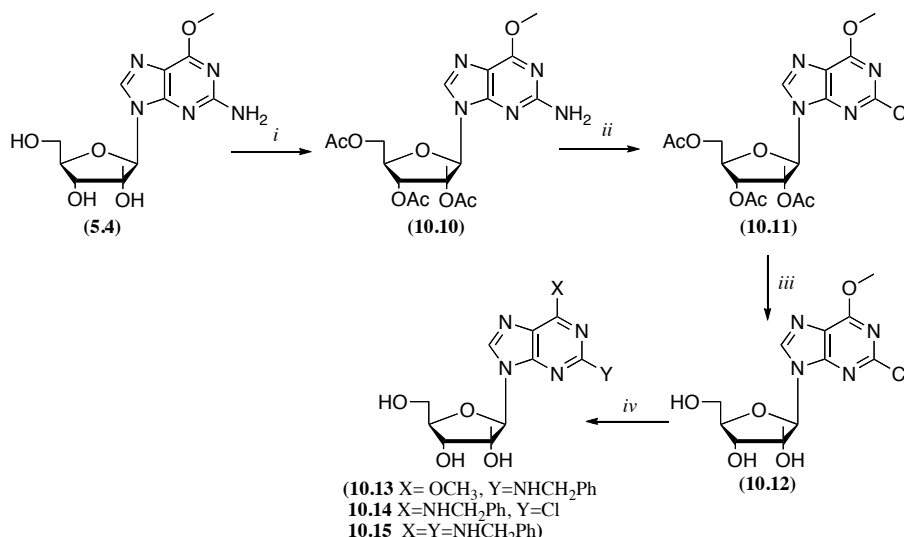
All attempts clearly suggest a relative instability of the starting material **10.4** during the reaction course. Due to the fact that direct coupling of unprotected nucleoside **10.4** and phosphorochloridate species was unsuccessful, and furthermore 2',3'-diol protection did not provide the desired product, it was decided to investigate a different route towards 2-*C*-benzylamine-2'-*C*-methylguanosine ProTides.

10.2.3 Synthesis of 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-β-methylguanosine via halogenation reaction.

During the synthesis of 6-*O*-methyl-2'-*C*-methylguanosine and related ProTides, it has been noticed that the introduction of the 6-*O*-methyl modification resulted in enhanced solubility of the parent nucleoside comparing to the unmodified 2'-*C*-methylguanosine **4.10**. The coupling reaction of nucleoside analogue **5.4** did not require 2',3'-protection and proceeded in good yields. We wondered whether the same effect would be observed in the

case of 2-*C*-benzylamine-2'-*C*-methylguanosine **10.4**, and therefore we decided to investigate the synthesis of 6-*O*-modified 2-*C*-benzylamine-2'-*C*-methylguanosine analogue.

The synthesis involved full protection of 6-*O*-methyl-2'-*C*-methylguanosine with acetyl protecting groups (Scheme 10.5). The 2',3',5'-protected nucleoside analogue **10.10** was obtained as a white solid in 66% yield.



Reagents and Condition: i) Et₃N, DMAP, Ac₂O, ACN, rt, 16 h; ii) AcCl, Bu₄NNO₂, DCM, -5 °C to 0 °C, 15 min; iii) NH₃/MeOH, 0 C to rt, 16 h; iv) PhCH₂NH₂, EtOH, reflux, 16 h.

Scheme 10.5. Synthesis of 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-methylguanosine.

As the introduction of C-2 bromo modification was low yielding, we decided to investigate a different approach. The attention was focused on C-2 chlorination reaction, as according to the literature it can provide much improved reaction yields comparing to the bromination.⁷

The reaction was performed according to the procedure described by Janeba *et al.*⁷ Thus, **10.10** was reacted with acetyl chloride in the presence of tetrabutylammonium nitrite in dry dichloromethane. After work up and column chromatography, pure **10.11** was obtained in 62% yield (Scheme 10.5). Methanolic ammonia mediated deprotection reaction resulted in formation of **10.12** in excellent yield (90%). Introduction of benzylamine functionality was performed in refluxing ethanol in the presence of benzylamine for 16 h. The resulting mixture consisted of three different products. During the reaction course partial displacement of 6-*O*-methyl group was observed. The major and the least lipophilic product was the desired 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-methylguanosine **10.13**. The other two products were

identified as 2-*C*-chloro-6-*C*-benzylamine-2'-*C*-methylguanosine **10.14** and **10.15** bearing benzylamine in both C2- and C6-positions (Scheme 10.5).

Although the reaction resulted in formation of desired **10.13**, due to the low reaction yield and very difficult purification of the last step, further optimization of the reaction conditions or an alternative route towards **10.13** would have to be investigated.

Reductive amination procedures or Buchwald type chemistry may be considered as an option.

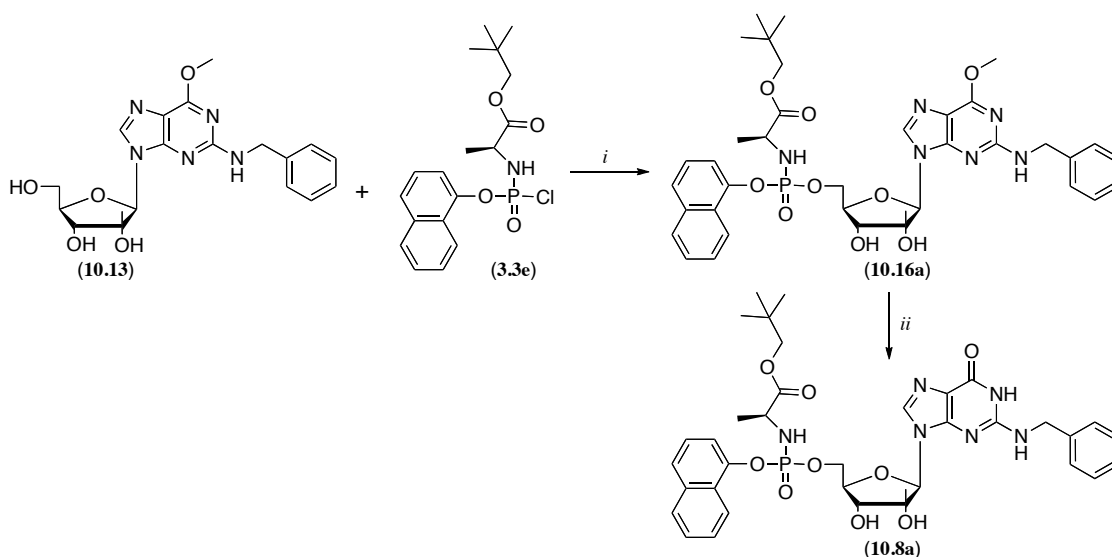
10.2.4 Synthesis of ProTides of 2-*C*-benzylamine analogues.

In order to synthesise α -naphthyl-L-alaninyl-*O*-2,2-dimethylpropyl phosphoramidate of **10.13**, the *t*BuMgCl-mediated coupling was applied.

The nucleoside analogue **10.13** was dissolved in dry THF and reacted with **3.3e** in the presence of the Grignard reagent. During the reaction course, formation of only one product was observed, as evidenced by the TLC analysis. After column chromatography on silica gel, using CHCl₃/MeOH (0 to 3% gradient) as eluent, pure **10.16a** was obtained as a white solid in 19% yield (Scheme 10.6).

At this point we decided to investigate, whether it would be possible to cleave the C6 methyl ether in order to obtain 2-*C*-benzylamine-2'-*C*-methylguanosine ProTide.

In the first instance the procedure reported by Moreau *et al.*⁸ was applied (Scheme 10.6).



Reagents and Condition: i) *t*BuMgCl, dry THF, rt, 16 h; ii) TMSCl, NaI, dry ACN, rt, 3 h.

Scheme 10.6. Synthesis of 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-methylguanosine and 2-*C*-benzylamine-2'-*C*-methylguanosine ProTides.

The cleavage of the C6 methyl ether was performed in dry acetonitrile in the presence of trimethylsilyl chloride (TMSCl) and sodium iodide (NaI), at ambient temperature. After 3 h of reaction course, TLC analysis revealed the presence of a new, more polar species. After purification using preparative TLC, pure **10.17a** was obtained as a white solid, in 29% yield.

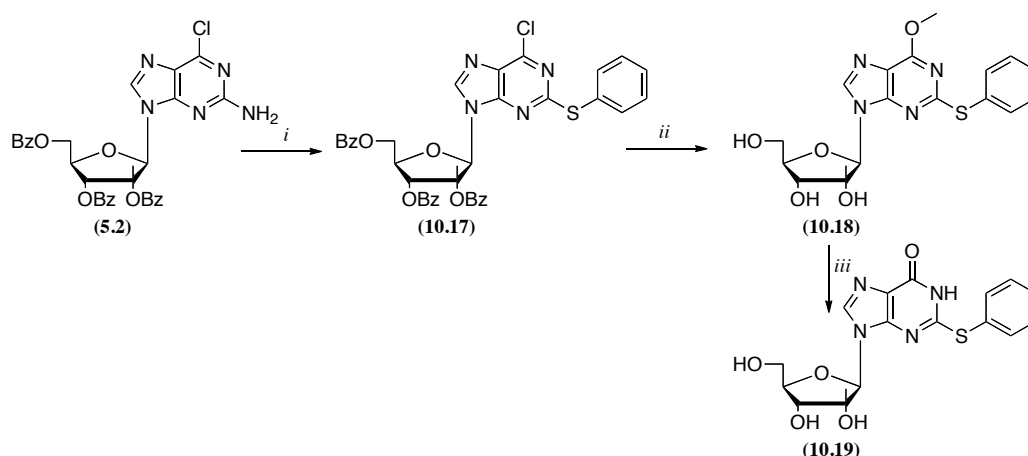
Biological evaluation of **10.16a** and **10.17a** will be reported and discussed in Section 10.6.

10.3 Synthesis of 2C-modified analogues – 2-C-thiophenyl analogues.

At the same time we were also interested in the preparation of 2-C-thiophenyl-2'-C-methylguanosine and its derivatives in order to investigate whether the presence of the hydrogen bond donor in the C2-position is crucial for the appropriate interaction in RdRp active site.

In the first instance the possibility of direct introduction of thiophenyl functionality at the C2-position was investigated (Scheme 10.7).

Reaction of 2-amino-6-chloro-9-(2'-C-methyl-β-D-ribofuranosyl)purine **5.2** with diphenyldisulfide and isopentyl nitrite in refluxing acetonitrile was carried out. Purification on silica gel, using CHCl₃ : MeOH (98:2) as eluent provided **10.17** contaminated with some impurities related to isopentyl nitrite, having same retention factor (Rf) as the desired product.



Reagents and Condition: i) diphenyldisulfide, isopentyl nitrite, dry ACN, reflux, 3 h; ii) NaOMe/MeOH, 0 C to rt, 16 h; iii) TMSCl, NaI, dry ACN, reflux 4 h.

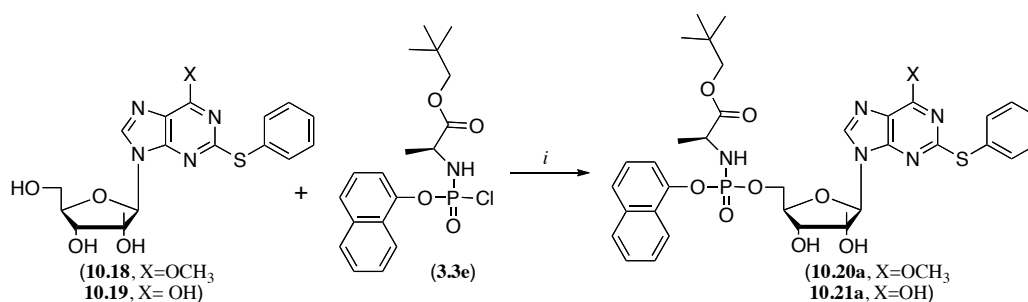
Scheme 10.7. Synthesis of 2-C-thiophenyl-6-O-methyl-2'-C-methylguanosine and 2-C-thiophenyl-2'-C-methylguanosine.

The crude **10.17** was submitted for the displacement and deprotection reaction using excess of sodium methoxide in methanol. After column chromatography, pure **10.18** was obtained in 44% yield (over 2 steps) as a white solid. Subsequent cleavage of the methyl ether in the presence of TMSCl and NaI, provided C6-keto analogue **10.19** in 42% yield.

Both 2-*C*-thiophenyl analogues **10.18** and **10.19** were submitted for the phosphoramidate coupling reaction.

10.3.1 Synthesis of ProTides of 2-*C*-thiophenyl analogues.

The coupling reactions were performed in dry THF using *t*BuMgCl coupling method. To facilitate comparison, both compounds were prepared as α -naphthyl-L-alanine-*O*-2,2-dimethylpropyl derivatives (Scheme 10.8).



Reagents and Condition: i) *t*BuMgCl, dry THF, rt, 16 h.

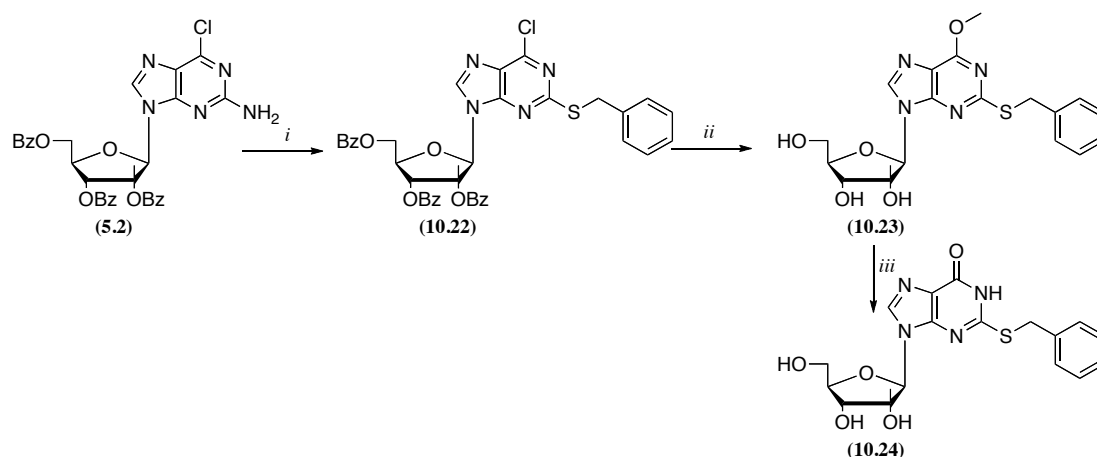
Scheme 10.8. Synthesis of ProTides of 2-*C*-thiophenyl analogues **10.18** and **10.19**.

The newly synthesised compounds were isolated as roughly equimolar mixtures of diastereoisomers at the phosphorus centre, evidenced by ³¹P NMR and HPLC.

Phosphoramidates **10.20a** and **10.21a** alongside with parent nucleosides **10.18** and **10.19** were submitted for the biological evaluation. The corresponding results will be reported and discussed in Section 10.6.

10.4 Synthesis of 2*C*-modified analogues – 2-*C*-thiobenzyl analogues.

In the next step, the exact sulphur-containing analogue of 2-*C*-benzylamine-2'-*C*-methylguanosine was prepared (Scheme 10.9).



Reagents and Condition: i) dibenzyl disulfide, isopentyl nitrite, dry ACN, reflux, 3 h; ii) NaOMe/MeOH, 0 °C to rt, 16 h; iii) TMSCl, NaI, dry ACN, reflux 4 h.

Scheme 10.9. Synthesis of 2-C-benzylamine-2'-C-methylguanosine.

Compounds **10.24** and **10.25** were prepared in analogous sequence as 2C-thiophenyl derivatives, using dibenzyl disulfide as a donor of thiobenzyl functionality (Scheme 10.9).

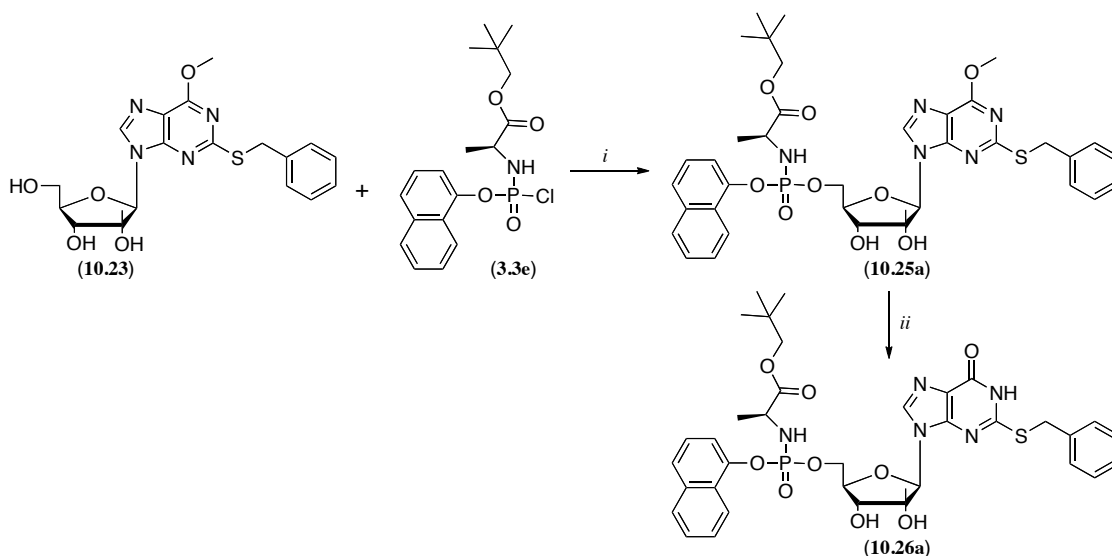
The 2-C-thiobenzyl-6-O-methyl-2'-C-methylguanosine **10.24** was obtained in 34% over two steps, and its guanine analogue **10.25** in 47% yield after the cleavage of the C6 methyl ether (Scheme 10.9).

The 2-C-thiobenzyl-6-O-methyl-2'-C-methylguanosine **10.24** was used in the subsequent phosphorylation reaction in order to obtain corresponding ProTide.

Both parent nucleosides **10.24** and **10.25** were evaluated against HCV in *in vitro* replicon assay, related data will be discussed in Section 10.6.

10.4.1 Synthesis of ProTides of 2-C-thiobenzyl analogues.

The coupling reaction was performed using the *t*BuMgCl-mediated method. To facilitate comparison, the compound was prepared as the α -naphthyl-L-alanine-*O*-2,2-dimethylpropyl analogue **10.25a** (Scheme 10.10).



Reagents and Condition: i) *t*BuMgCl, dry THF, rt, 16 h; ii) TMSCl, NaI, dry ACN, rt, 2 h.

Scheme 10.10. Synthesis 2-*C*-thiobenzyl ProTides of **10.25a** and **10.26a**.

As the procedure used for the cleavage of the methyl ether was found to be successful on the ProTide level, compound **10.26a** was prepared from **10.25a** by treatment with TMSCl and NaI. After purification on preparative TLC, pure **10.26a** was obtained in 34% yield.

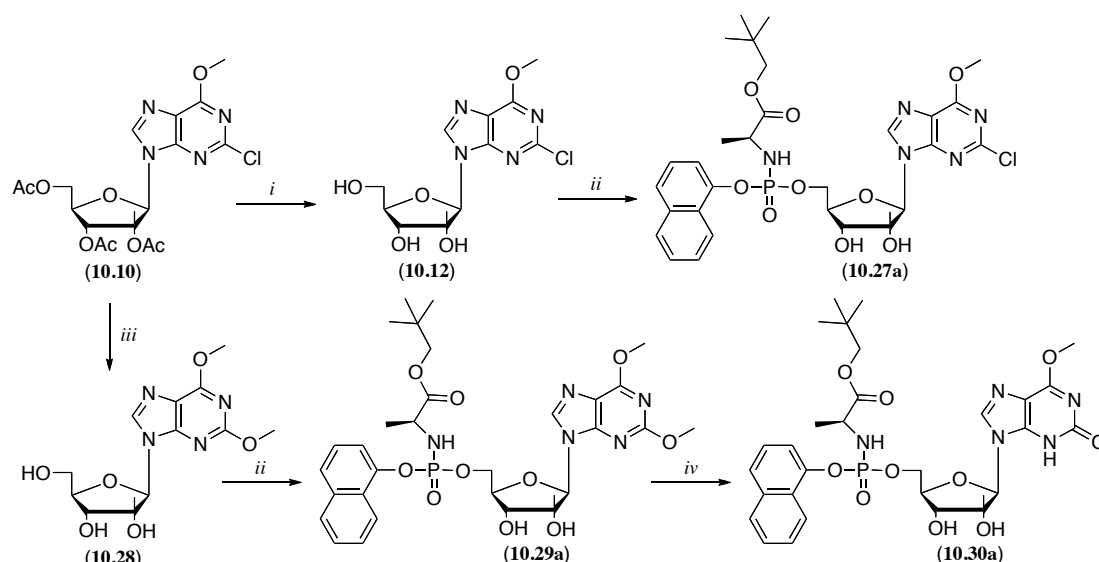
The newly synthesised compounds were isolated as roughly 1:1 mixtures of diastereoisomers at the phosphate, evidenced by ^{31}P NMR and HPLC.

Phosphoramidates **10.25a** and **10.26a** were evaluated for their anti-HCV activity in cell-based replicon assay. The corresponding data will be reported and discussed in Section 10.6.

10.5 Synthesis of ProTides of other 2-*C*-modified analogues.

During the synthetic pathway towards 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-methylguanosine **10.13**, a synthetic intermediate bearing chlorine in C2-position **10.11** was found to be of interest. It was decided to deprotect **10.12** and to apply the ProTide approach in order to investigate the influence of the C2-chloro substituent on the anti-HCV activity.

The deprotection reaction, carried out in methanolic ammonia, provided 2-*C*-chloro-6-*O*-methyl-2'-*C*-methylguanosine **10.12** in excellent 90% yield (Scheme 10.11). After coupling with α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate **3.3e**, **10.27a** was obtained in 19% yield (Scheme 10.11). Due to the low amount of **10.27a** the attempt of cleaving C6 methyl ether was not performed.



Reagents and Condition: i) NH_3/MeOH , 0 °C to rt, 16 h; ii) $t\text{BuMgCl}$, dry THF, rt, 16 h; iii) NaOMe/MeOH , 0 °C to rt, 3 days; iv) TMSCl , NaI , dry ACN, rt, 3 h.

Scheme 10.11. Synthesis of 2-C-benzylamine-2'-C-methylguanosine.

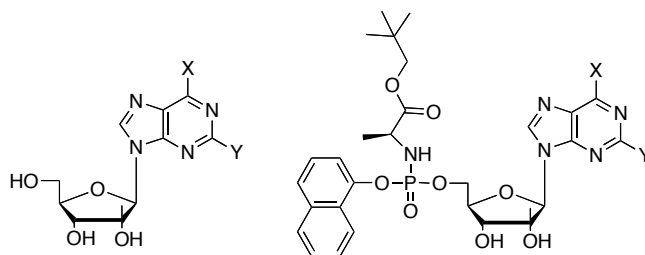
It was also interesting to see, whether the substitution of C2-chloro with a methoxy moiety will have an impact on biological activity. The displacement-deprotection reaction was carried out in an excess of sodium methoxide in methanol (Scheme 10.11). The best results were obtained using 6 mol equivalents of sodium methoxide added portionwise over 3 days reaction course. Because the desired product and C2-chloro analogue have exactly the same R_f on TLC, the progress of the reaction was monitored by NMR. Small samples were collected from the reaction mixture and after work up with Amberlite H^+ , proton NMR spectra were recorded. After full conversion of **10.10**, as evidenced by proton NMR, purification was performed and **10.28** was obtained in 86% yield (Scheme 10.11).

The coupling reaction with the appropriate phosphorochloridate **3.3e** gave **10.29a** in 17% yield (Scheme 10.11). Subsequent reaction of C6 methyl ether cleavage performed in the presence of TMSCl and NaI , provided C6-modified xanthosine type derivative **10.30a** in 47% (Scheme 10.11).

All synthesised phosphoramidates **10.27a**, **10.29a** and **10.30a** were isolated as a diastereomeric mixtures (1:1) at the phosphorus centre.

10.6 Biological evaluation of 2-C-modified analogues and corresponding ProTides.

All newly synthesised C2-modified nucleosides and corresponding aryloxyphosphoramidates were evaluated in *in vitro* HCV replicon assay by Inhibitex Inc., (Table 10.3). 2'-C-methylguanosine **4.10**, 6-O-methyl-2'-C-methylguanosine **5.4** and clinical candidate **INX-189** were used as references.



| Cpd | X | Y | AA | Ester | ClogP | EC ₅₀ [μM] | CC ₅₀ [μM] |
|----------------|------------------|----------------------|-------|--------------------|-------|-----------------------|-----------------------|
| 4.10 | OH | NH ₂ | - | - | -2.6 | 3.5 | >100 |
| 5.4 | OCH ₃ | NH ₂ | - | - | -0.6 | 4.6 | >100 |
| INX-189 | OCH ₃ | NH ₂ | L-Ala | 2,2-dimethylpropyl | 3.1 | 0.01 | 7 |
| 10.4 | OH | NHCH ₂ Ph | - | - | -0.4 | >10 | >100 |
| 10.8a | OH | NHCH ₂ Ph | L-Ala | 2,2-dimethylpropyl | 3.6 | 4.9 | >100 |
| 10.13 | OCH ₃ | NHCH ₂ Ph | - | - | 1.6 | >100 | >100 |
| 10.16a | OCH ₃ | NHCH ₂ Ph | L-Ala | 2,2-dimethylpropyl | 5.6 | >40 | >100 |
| 10.18 | OCH ₃ | SPh | - | - | 2.3 | >100 | >100 |
| 10.20a | OCH ₃ | SPh | L-Ala | 2,2-dimethylpropyl | 6.2 | 13 | 25 |
| 10.19 | OH | SPh | - | - | 0.4 | >100 | >100 |
| 10.21a | OH | SPh | L-Ala | 2,2-dimethylpropyl | 4.3 | 1.4 | >100 |
| 10.23 | OCH ₃ | SCH ₂ Ph | - | - | 2.0 | 42 | >100 |
| 10.25a | OCH ₃ | SCH ₂ Ph | L-Ala | 2,2-dimethylpropyl | 6.0 | 12 | 16 |
| 10.24 | OH | SCH ₂ Ph | - | - | 0.2 | 35 | >100 |
| 10.26a | OH | SCH ₂ Ph | L-Ala | 2,2-dimethylpropyl | 4.1 | 0.73 | 58 |
| 10.12 | OCH ₃ | Cl | - | - | -0.02 | >100 | >100 |
| 10.27a | OCH ₃ | Cl | L-Ala | 2,2-dimethylpropyl | 3.9 | 2 | 28 |
| 10.28 | OCH ₃ | OCH ₃ | - | - | 0.22 | >100 | >100 |
| 10.29a | OCH ₃ | OCH ₃ | L-Ala | 2,2-dimethylpropyl | 4.2 | 16 | 62 |
| 10.30a | OCH ₃ | OH | L-Ala | 2,2-dimethylpropyl | 1.5 | 0.8 | >100 |

AA: Amino acid; ClogP values calculated using ChemOffice Ultra 11.0; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

Table 10.3. Summary of ant-HCV activity of C2-modified nucleoside analogues and their corresponding ProTides.

As can be seen in Table 10.3, most of the parent nucleosides bearing modifications at C6- and C2-position at the same time were completely inactive in the replicon based assay (Entries **10.12**, **10.13**, **10.18**, **10.29**, $EC_{50} > 100 \mu M$). The only exception was compound **10.23** – 2-*C*-thiobenzyl-6-*O*-methyl-2'-*C*-methylguanosine analogue which displayed some anti-HCV activity at 40 μM concentration. Compounds, which were modified only at the C2-position, were found to exhibit poor to moderate activity in cell based assay (**10.24**, $EC_{50} = 35 \mu M$). The anti-HCV activity of 2-*C*-benzylamin-6-*O*-methyl-2'-*C*-methylguanosine **10.4** was found to be $>10 \mu M$. All parent nucleosides were non-toxic in the replicon assay up to 100 μM concentration.

Looking at compounds at the ProTide level, it can be clearly noticed that the application of aryloxyphosphoramidate technology to the inactive or poorly active parent nucleosides resulted in compounds with anti-HCV activity in low micromolar and submicromolar range. The least active compound across the whole family was 2-*C*-benzylamine-6-*O*-methyl-2'-*C*-methylguanosine ProTide **10.16a**, which activity was greater than 40 μM . In contrast its C6-keto analogue **10.8a** displayed EC_{50} value of 4.9 μM , being >10 -times more potent than **10.16a** but at the same time ca. 500-fold less potent than the clinical candidate **INX-189**. This result was somewhat disappointing as the compound showed the best fit in the active site of RdRp (according to the molecular modelling). It would be of interest to synthesise analogues bearing different substituents on the benzyl ring to see whether the anti-HCV activity can be improved. The compound was non-toxic up to 100 μM concentration. No clear correlation between ClogP values and anti-HCV activity was found.

Compounds **10.20a** (2-*C*-thiophenyl-6-*O*-methyl-2'-*C*-methylguanosine ProTide) and **10.25a** (2-*C*-thiobenzyl-6-*O*-methyl-2'-*C*-methylguanosine ProTide) were found to exhibit similar activity $EC_{50} = 13$ and 12 μM , respectively. Both compounds displayed some toxicity in the replicon assay ($CC_{50} = 25$ and 16 μM , respectively). Considering the anti-HCV activity of these compounds, their selectivity indexes would be ca. 2 for **10.20a** and 1 for **10.25a**, and would make them one of the most toxic across reported series of ProTides. Interestingly, their C6-keto analogues **10.21a** and **10.26a** were found to be much more potent ($EC_{50} = 1.4$ and 0.73 μM , respectively) and considerably less toxic ($CC_{50} > 100$ and = 58 μM , respectively). The compound **10.26a** with an EC_{50} value of 0.07 μM was found to be the most active across the whole family, being ca. 50-times more potent than the parent nucleoside **10.24**,

and ca. 70-times less active than the clinical candidate **INX-189**. High potency of these compounds proved, that the presence of the hydrogen bond donor functionality at the C2-position is not crucial for the anti-HCV activity. Furthermore these compounds exhibited much improved HCV inhibitory activity comparing to the compound bearing amino group in the C2 ($EC_{50} = 4.9 \mu\text{M}$ vs. $0.73 \mu\text{M}$, for **10.8a** and **10.26a**, respectively).

The compound **10.27a** bearing C6-methoxy and C2-chloro substituents was active at $2 \mu\text{M}$ concentration, while its analogue possessing methoxy functionality in both C6- and C2-position was poorly active $EC_{50} = 58 \mu\text{M}$. Interestingly, xanthosine type analogue **10.30a** displayed anti-HCV activity at $0.8 \mu\text{M}$ concentration, being as potent as **10.26a** ($EC_{50} = 0.73 \mu\text{M}$), and non-toxic up to $100 \mu\text{M}$ concentration.

Reported results clearly indicate that presence of modifications at both C6- and C2-position greatly decreases anti-HCV activity of synthesised ProTides. The most probable explanation is that the active compounds require C6-keto functionality in order to display HCV inhibitory activity. The presence of the C2-substituents may prevent ADA-mediated hydrolysis of C6-modifications and as a result lower or no antiviral activity has been observed. To support this theory ADA assays on parent nucleosides **10.18** and **10.28** were performed (Figure 10.1).

Experiments were performed according to the procedure described in Chapter 5, Section 5.2.3. As can be seen in Figure 10.1, compounds **10.18** and **10.28** were found not to be suitable substrates for adenosine deaminase. No spectral change (shift in λ_{max}) was observed during 10 h of experiment (Figure 10.1).

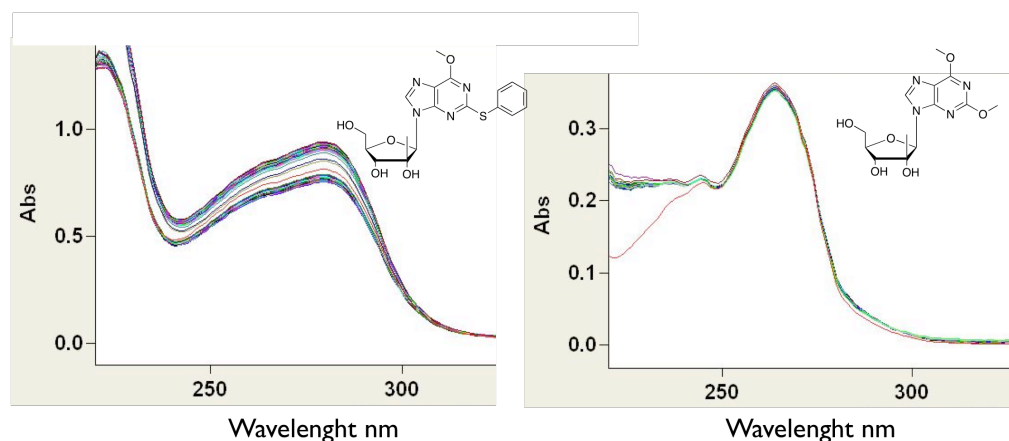


Figure 10.1. Spectral change during ADA-mediated hydrolytic removal of alkyl group from **10.18** and **10.28**.

The obtained results may to some extent explain lack of activity (or significantly decreased activity) of 6-*O*-methyl modified analogues and ProTides.

10.7 Conclusions.

In summary, a series of novel 2-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine and 2-*C*-modified-2'-*C*-methylguanosine analogues and their aryloxyphosphoramidates has been synthesised. During biological evaluation it was found that compounds bearing modifications in C6- and C2-position were significantly less active than their corresponding C6-keto analogues. These findings were supported by the ADA-mediated hydrolytic assays.

The best results were obtained for 2-*C*-thiophenyl (**10.21a**), 2-*C*-thiobenzyl (**10.26a**) and xanthosine (**10.30a**) analogues.

Given the importance of finding a new pharmacophore for the anti-HCV drug development and promising biological results obtained, further work should be carried out in order to discover the full potential of these new promising family of compounds, particularly in the 6-keto series.

References:

1. Choi JY, Guengerich FP. Analysis of the effect of bulk at N2-alkylguanine DNA adducts on catalytic efficiency and fidelity of the processive DNA polymerases bacteriophage T7 exonuclease⁻ and HIV-1 reverse transcriptase. *J Biol Chem* **2004**, 18, 19217-19229.
2. Hildebrand C, Sadoli D, Focher F, Joseph G, Ciarrocchi G, Spadari S, Wright G. Structure activity relationships of N²-substituted guanines as inhibitors of HSV1 and HSV2 thymidine kinase. *J Med Chem* **1990**, 33, 203-206.
3. Focher F, Hildebrand C, Freese S, Ciarrocchi G, Noonan T, Sangalli S, Brown N, Spadari S, Wright G. N²-Phenyldeoxyguanosine: A novel selective inhibitor of Herpes Simplex thymidine kinase. *J Med Chem* **1988**, 31, 1498-1500.
4. Wright GE, Baril EF, Brown VM, Brown NC. Design and characterization of N²-arylamino purines which selectively inhibit replicative DNA synthesis and replication-specific DNA polymerases: guanine derivatives active on mammalian DNA polymerase alpha and bacterial DNA polymerase III. *Nucleic Acid Res* **1982**, 10, 4431-4440.
5. a) Molecular modelling performed by dr. A. Brancale and dr. N. Zonta at School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff.
The HCV polymerase model build according to the literature:
b) Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhta B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, Cortese R, De Francesco R, Eldrup AB, Getty KL, Hou DS, LaFemina RL, Ludmerer SW, MacCoss M, McMaster DR, Stahlhut MW, Olsen DB, Hazuda DJ, Flores OA. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogues that inhibit hepatitis C virus replication *in vitro*. *J Biol Chem* **2003**, 278, 49164.
6. Trivedi BK. Studies toward synthesis of C2-substituted adenosines: An efficient synthesis of 2-(phenylamino)adenosine (CV-1808). *Nucleosides and Nucleotides* **1988**, 7(3), 393-402.
7. Janeba Z, Francom P, Robins MJ. Efficient syntheses of 2-chloro-2'-deoxyadenosine (Cladribine) from 2'-deoxyguanosine. *J Org Chem* **2003**, 68, 989-992.
8. Moreau C, Wagner GK, Weber K, Guse AH, Potter BVL. Structural determinants for N1/N7 cyclization of nicotinamide hypoxanthine 5'-dinucleotide (NHD⁺) derivatives by ADP-ribosyl cyclase from *Aplysia californica*: Ca²⁺- Mobilizing activity of 8-

substituted inosine 5'-diphosphoribose analogues in T-lymphocytes. *J Med Chem* **2006**, 49, 5162-5176.

CHAPTER ELEVEN

11.1 Main conclusions and perspectives.

Each project was concluded in the appropriate section of each Chapter. In the following the main conclusions are summarised.

Design, synthesis and biological evaluation of 2'-*C*-methylguanosine ProTides resulted in almost every case in greatly improve anti-HCV activity comparing to the parent nucleoside, often by 10-30-fold and sometimes up to 1000-fold. Extensive structure activity relationships studies resulted in very potent compounds, suitable for further preclinical development. Compounds were found to be stable in different media including human plasma. Intestinal and liver S9 stability data, followed by the experiments in simulated gastric and intestinal fluids gave an indication that oral administration would be feasible.

Further development of ProTides bearing modification in the base unit of the nucleoside analogue, resulted in identification of the 6-*O*-methyl-2'-*C*-methylguanosine α -naphthyl-L-alanine-*O*-2,2-dimethypropyl ProTide showing the biggest promise. This particular compound was further progressed into clinical development as **INX-189**. **INX-189** is currently in the phase IIa clinical development.

In an effort to obtain achiral prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine, a new phosphorodiamidate approach was investigated. As a result compounds exhibiting nM activity in HCV replicon assay were identified. This is the first time, when application of a phosphorodiamidate approach to the nucleoside analogue was successful and lead to the suitable candidates for an **INX-189** back up programme.

Additionally, a novel family of oxazaphosphorine type prodrugs of 6-*O*-methyl-2'-*C*-methylguanosine was reported. Compounds were found to be not active in the replicon based assay, however their mode of action is based on cytochrome P₄₅₀-mediated oxidation and therefore to fully validate the potential of this class of molecules, further testing in more appropriate conditions (presence of CYP inducing agents) is required.

A series of novel 8-*C*-modified-6-*O*-methyl-2'-*C*-methylguanosine nucleoside analogues and their aryloxyphosphoramidate, and diamidate prodrugs has been synthesised, resulting in identification of 8-*C*-iodo substituted analogues as a new promising anti-HCV agents with nanomolar activity. Further biological evaluation

revealed that the 8-C-iodo substituent act as a promoiety with eventual release of 2'-C-methylguanosine monophosphate.

During the design, synthesis and biological evaluation of novel 2-C-modified-6-O-methyl-2'-C-methylguanosine and 2-C-modified-2'-C-methylguanosine analogues and their corresponding ProTides, 2-C- thiophenyl, 2-C-thiobenzyl and xanthosine analogues were identified as new interesting anti-HCV agents. Given the importance of finding new a pharmacophore for the anti-HCV drug development and promising biological results obtained, further work on this particular family of analogues should be carried forward.

CHAPTER TWELVE

EXPERIMENTAL PROCEDURES

12.1 General Experimental Details.

Glassware

All glassware was dried in the oven at 130 °C for several hours and allowed to cool down.

Solvents and Reagents

All solvents and reagents commercially available were used without any further purification.

The following solvents were purchased as anhydrous: acetone, acetonitrile (ACN), chloroform (CHCl_3), dichloromethane (DCM), diethyl ether (Et_2O), N,N-dimethylformamide (DMF), ethanol (EtOH), methanol (MeOH), pyridine, tetrahydrofuran (THF).

Thin Layer Chromatography

The reactions were analysed by Thin Layer Chromatography (TLC). Aluminium backed TLC plates, precoated with silica gel (60 F₂₅₄, 0.2 mm) were purchased from Merck Kieselgel. Separated components were visualized using ultra violet light (245 and 366 nm).

Preparative TLC plates (20 x 20 cm, 500-2000 μM) were also purchased from Merck.

Column Chromatography (CC)

Column chromatography was performed using normal phase Silica gel 35-70 μ , 60A (Fluka/Fisher) as a stationary phase. Glass columns were slurry packed in the appropriate eluent under gravity. Samples were applied as concentrated solutions in the same eluent, or pre absorbed onto silica gel. The fractions containing the product were analyzed by TLC, and then combined together and the solvent removed under vacuum.

Nuclear Magnetic Resonance (NMR)

^1H , ^{13}C , ^{31}P spectra were recorded on a Bruker Avance 500 spectrometer (500, 125, 202 MHz, respectively), at 25 °C. ^{31}P NMRs are reported in units of δ relative to 85% phosphoric acid as the external standard. Spectra were calibrated to the residual peak of the deuterated solvent. In ^1H NMR, ^{13}C NMR, ^{31}P NMR shifts are given in parts per million (ppm) and rounded to two decimal places.

The following abbreviations are used in the NMR signals assignment: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *m* (multiplet).

Coupling constants (*J*) are measured in Hertz and rounded to one decimal place. Unless otherwise stated, coupling constants are three-bond couplings between two hydrogen atoms. Other couplings are marked with a subscript, according to the type of coupling e.g.: $^2J_{\text{C-O-P}}$ (two bond coupling between carbon and phosphorus).

High Performance Liquid Chromatography (HPLC)

Analytical and semi-preparative experiments were carried out on a Varian ProStar (LC Work Station - Varian ProStar 335LC detector, Varian fraction collector - model 701, ProStar 201 delivery system, using Varian Pursuit XRs 5C18 (150 x 4.6 mm) as an analytical column and Varian Puruit XRs 5C18 (150 x 21.2 mm) as semi-preparative column. Used software was Galaxie Chromatography Data System. Elution were performed using:

- Mobile phase water/ acetonitrile in gradient ($\text{H}_2\text{O}/\text{ACN}$: 0% to 100% of ACN in 30 minutes) – System 1.
- Mobile phase water/ methanol in gradient ($\text{H}_2\text{O}/\text{MeOH}$: 0% to 100% of ACN in 30 minutes) – System 2.

Mass Spectrometry (MS)

High (HRMS) and low resolution mass spectrometry was performed as a service by Cardiff University, using electrospray.

Elemental analysis (CHN)

Elemental analysis was performed as a service by MEDAC Ltd., Surrey.

Enzymatic Assays:**Carboxypeptidase Y Assay**

5mg of appropriate phosphoramidate was dissolved in 200 μ l of deuterated acetone and 400 μ l of TRIZMA buffer (pH 7.6) was added. A ^{31}P -NMR was conducted at this stage as a reference ($t=0$). To this mixture 0.3mg of Carboxypeptidase Y (purchased from SIGMA, > 50unit/mg, EC 3.4.16.1) dissolved in 200 μ l of TRIZMA buffer was added. ^{31}P -NMR of the reaction mixture was carried out every 7 minutes for 14 hours, at room temperature.

Adenosine Deaminase Assay

Stock solutions of appropriate nucleoside analogue were dissolved in phosphate buffer (pH 7.5, 0.05M) to a final concentration of 44 μ M. To a solution of adenosine deaminase from calf intestinal mucosa (solution in 50% glycerol, 0.01M potassium phosphate, pH 6.0, Sigma-Aldrich) phosphate buffer (pH 7.5, 0.05M) was added to a final volume of 2.0 ml.

For each assay, 1ml of 44 μ M solution of the appropriate nucleoside analogue was placed in a 1cm UV cuvet, at 25 C, and UV spectrum was recorded over the range 400 to 220 nm. A portion of enzyme solution (30 to 200 μ M) was added, and spectra were recorded in 1 min intervals for 30 min, and after that time in 10 or 30 min intervals for 8 h.

Cell Lysate Assay

The cell lysate experiments were performed using Huh7 human hepatoma cells (10^7 cells) lysate, which was added and incubated with the appropriate aryloxyphosphoramidate or phosphorodiamidate in deuterated solvent (D_2O or d_6 -acetone) at 37 °C. ^{31}P NMR spectra were recorded every hour for 14 hours.

Blank experiment (ProTide in deuterated solvent) was recorded prior lysate addition.

Human liver microsomes assay

Appropriate cyclic phosphoramidate was incubated in the presence of human liver microsomes (purchased from Sigma). Incubation was carried out without the presence of NADPH co-factor. The incubation mixture (final volume of 600 μ L) contained: 90

μL of microsomal protein, 235 μL of Tris buffer (pH 7.5) and appropriate cyclic phosphoramidate (500 μM concentration in Tris buffer, 75 μL) and 200 μL of D_2O .

The reaction was followed by the ^{31}P NMR at 37 °C, for 14 h. Spectra were recorded in 7 min intervals with blank spectra of compound. The blank spectrum was recorded in the Tris buffer prior the microsomes addition.

Biological Testing

Biological testings (unless stated otherwise) were performed by John Vernachio's group, Inhibitex Inc., USA.

12.2 Standard Procedures.

Standard procedure 1: Preparation of phosphorodichloridate species.

Phosphorus oxychloride (1.0 mol equivalents) and the appropriate naphhol (1.0 mol equivalent) were stirred in anhydrous diethyl ether. Anhydrous triethylamine was added (1.0 mol equivalent) at -78 °C and after 25 minutes, the solution was allowed to warm to ambient temperature. Progress of the reaction was monitored by the ^{31}P NMR. After reaction completion, triethylamine hydrochloride salts were filtered off and the solvent was removed under reduce pressure to give a clear pale yellow oil.

Standard procedure 2a: Preparation of amino acid ester hydrochloride salts.

To a stirred solution of appropriate alcohol (15,0 mol equivalents) at 0 °C under argon atmosphere, thionyl chloride (2,0 mol equivalents) was added. The reaction mixture was stirred at 0 °C for 30 minutes and after that time slowly allowed to warm to ambient temperature. The appropriate amino acid (1,0 mol equivalent) was added and the mixture was heated at 70 °C overnight. After that time, solvents were removed under vacuum (the last traces of solvents were removed by co-evaporation). Pure product was obtained by precipitation from diethyl ether, as a white solid of hydrochloride salt.

Standard procedure 2b: Preparation of amino acid ester sulfonate salts.

To the amino acid (1 mol equivalent) in toluene, was added: appropriate alcohol (5 to 15 mol equivalents) and *para*-toluene sulfonic acid (1.1 mol equivalents). The mixture was heated at reflux overnight using Dean-Stark apparatus. After that time solvents were removed under reduce pressure. The amino acid ester was then precipitated either from diethyl ether or ethyl acetate (0 °C). Pure product was obtained as a white solid of *p*- toluene sulfonate salt.

Standard procedure 2c: Preparation of Boc amino acid esters.

To the Boc-protected amino acid (1 equiv.) in DCM (20 ml/g of amino acid) the alcohol (1.2 to 2 equiv.), DCC (1 equiv.) and DMAP (0.1 equiv.) were added at room temperature. After being stirred overnight, the solvent was removed under reduce pressure and the residue was purified on silica gel (Hexane/AcOEt 9:1) to afford the pure Boc-amino acid ester.

Standard procedure 2d: Boc amino acid deprotection.

To the Boc-amino ester (1 eq) in AcOEt (35ml/g of Boc-amino ester), *p*TSA (1 eq) was added. The mixture was stirred for 2 hours at 65 °C and the solvent was removed under reduce pressure to afford the pure amino ester *p*TSA salt. The latter can crystallise in AcOEt at 0 °C or from MeOH/Et₂O.

Standard procedure 3: Preparation of phosphorochloridate species.

Aryl phosphorodichloridate (1 mol equivalents) and the appropriate amino acid ester (1 mol equivalent) were suspended in anhydrous dichloromethane. Anhydrous triethylamine (2 mol equivalents) was added dropwise at -78 °C and after one hour, the reaction was left to rise to room temperature and stirred for 2 to 4 hours. The formation of phosphorochloridate was monitored by ³¹P NMR. The solvent was removed under vacuum and the crude residue was purified by a quick column chromatography on silica gel (Hexane/EtOAc 1:1).

Standard procedure 4: Preparation of 2',3'-isopropylidene protected 2'-C-methylguanosine ProTides.

2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine (1 mol equivalent) was dissolved in anhydrous THF, solution of *t*BuMgCl (1M in THF, 2 mol equivalent) was added dropwise and stirred for 20 min. Appropriate phosphorochloridate (2 mol equivalent) in anhydrous THF was added very slowly to the reaction mixture. The reaction was stirred overnight at room temperature under argon atmosphere. After that time solvent was removed under reduced pressure. The foamy residue was purified by column

chromatography in a gradient (CHCl₃/MeOH 98:2 to CHCl₃/MeOH 92:8) to afford the pure desired product.

Standard procedure 5: Deprotection of 2',3'-isopropylidene protected 2'-C-methylguanosine ProTides.

The protected phosphoramidate was dissolved in a 60% acetic acid in water (~10ml / 200 mg) and heated at 95 °C overnight. The solvent was then removed under reduced pressure. Residue was purified by column chromatography using as an eluent CHCl₃/MeOH (9:1), to give the desired product as a white solid.

Standard procedure 6: Preparation of aryloxyphosphoramidates (NMI method).

To the solution/suspension of appropriate nucleoside analogue (1 mol equivalent) in dry THF, phosphorochloridate (3 mol equivalent) in THF was added, followed by the addition of *N*-methylimidazole (5 mol equivalent) under argon atmosphere. The reaction mixture was stirred overnight at ambient temperature and after that time, the solvent was removed under reduce pressure. To remove the residues of *N*methylimidazole, the crude phosphoramidate was dissolved in chloroform (or dichloromethane) and washed 3 times with hydrochloric acid (HCl 0.1N). The organic layer was then dried over sodium sulfate and evaporated to dryness under reduce pressure. The residue was then purified on silica gel using CHCl₃/MeOH (0 to 5%, gradient) as an eluent, to give the pure phosphoramidate as a white solid.

Standard procedure 7: Preparation of aryloxyphosphoramidates (tBuMgCl method).

To the solution/suspension of appropriate nucleoside analogue (1 mol equivalent) in dry THF, solution of *t*BuMgCl (1M in THF, 2 mol equivalents) was added dropwise and stirred for 30 min. The appropriate phosphorochloridate (2 mol equivalents) in anhydrous THF (1M solution) was added very slowly to the reaction mixture. The reaction was stirred overnight at ambient temperature under argon atmosphere. After that time solvent was removed under reduced pressure. The foamy residue was

purified by column chromatography in a gradient (CHCl₃ to CHCl₃/MeOH 95:5) to afford the pure desired product.

Standard procedure 8: Preparation of 5'-cyclic phosphoramidates.

2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-D-ribofuranosyl) purine (1 mol equivalent) was dissolved in anhydrous pyridine and appropriate cyclic phosphorochloridate (1 mol equivalent) in anhydrous pyridine (1M solution) was added very slowly to the reaction mixture. The reaction was stirred overnight at room temperature under argon atmosphere. After that time solvent was removed under reduced pressure. The foamy residue was purified by column chromatography using in CHCl₃/MeOH or CHCl₃/MeOH as eluent, afford the pure desired product.

Standard procedure 9: Preparation of N-(2-nitrophenylsulfonyl)-amino acid esters.

2-Nitrosulfonylbenzyl chloride (1.0 mol equivalent) was added to an ice-cold solution of the appropriate amino acid ester salt (1.0 mol equivalent) and triethylamine (2.3 mol equivalent) in dry DCM (70ml/10.0 mmol). The mixture was allowed to warm to ambient temperature and stirred overnight. The resulting solution was washed with water (3x), dried over anhydrous MgSO₄ and concentrated in vacuo. The resulting *N*-(2-nitrophenylsulfonyl)-amino acid ester was used without further purification.

Standard procedure 10: Preparation of N-(3-hydroxypropyl)-N-(2-nitrophenylsulfonyl)-amino acid esters.

Cesium carbonate (1.0 mol equivalent) was added to a solution of the *N*-(2-nitrophenylsulfonyl)-amino acid ester (1.0 mol equivalent) in DMF (45 ml/10 mmol). The reaction mixture was heated to 60 °C and then 3-bromopropan-1-ol (1,5 mol equivalent) was added dropwise with stirring. The reaction mixture was stirred overnight at 60 °C. After that time, the mixture was diluted with water and extracted 3x with ethyl acetate. The combined organic phases were dried over anhydrous Na₂SO₄ and concentrated. The resulting *N*-(3-hydroxypropyl)-*N*-(2-

nitrophenylsulfonyl)-amino acid ester was purified by column chromatography using EtOAc/petroleum ether (8:2) as an eluent.

Standard procedure 11: Preparation of N-(3-hydroxypropyl)-amino acid esters.

Potassium carbonate was added to a solution of the resulting *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulfonyl)-amino acid ester (1.0 mol equivalent) and thiophenol (1.1 mol equivalent) in dry acetonitrile (15 ml/10 mmol). The reaction mixture was stirred at ambient temperature overnight. After that time the reaction mixture was concentrated under reduced pressure and the residue was taken up in diethyl ether. Hydrochloric acid solution (1N) was added and the mixture was stirred for 10 min (till all salts are dissolved). The organic layer was removed and washed with water. The combined aqueous layers were washed with diethyl ether followed by basification with solid K_2CO_3 (3.25 mol equivalent). The product was extracted with DCM or EtOAc several times. Organic phases were combined, dried over Na_2SO_4 , evaporated to dryness and purified on silica gel, using $CHCl_3/MeOH$ (9:1) as an eluent..

Standard procedure 12: Preparation of cyclic phosphorochloridates.

N-(3-hydroxypropyl)-amino acid ester (1.0 mol equivalent) was dissolved in dry $CHCl_3$ (0.5 ml/2.5 mmol) and Et_3N (1.0 mol equivalent) was added. $POCl_3$ (1.0 mol equivalent) was dissolved in dry $CHCl_3$ (2.5 ml/2.5 mmol) and the solution was cooled to $-15\text{ }^{\circ}C$. A solution of amino acid was added dropwise to the $POCl_3$ mixture, followed by the addition of further quantity of Et_3N (1.0 mol equivalent) in dry $CHCl_3$ (0.5 ml/ 2.5 mmol). The temperature was kept below $0\text{ }^{\circ}C$ all the time, and the reaction mixture was stirred at $0\text{ }^{\circ}C$ for 18 hours. After that time solvent was removed under reduced pressure at $35\text{ }^{\circ}C$. The crude mixture was purified on silica gel (flash chromatography), using EtOAc/hexane (7:3) as an eluent.

Standard procedure 13a: Preparation of symmetrical phosphorodiamidates.

To a suspension of the nucleoside (1.0 mol equivalent) in dry tetrahydrofuran, triethylamine (1.0 mol equivalent) was added. After stirring for 30 min at ambient temperature, phosphorus oxychloride (1.0 mol equivalent) was added dropwise at -78

°C. The reaction mixture was stirred for 30 min at -78 °C and then allowed to warm to ambient temperature over 30 min (Progress of the reaction was followed by ^{31}P NMR). After reaction completion, anhydrous dichloromethane was added, followed by amino acid ester (3-5 mol equivalent) and triethylamine (6-10 mol equivalent) at -78 °C. After stirring at ambient temperature for 16-20 h, the reaction mixture was evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as eluent a gradient of methanol in dichloromethane.

Standard procedure 13b: Preparation of asymmetrical phosphorodiamidates (POCl₃ route).

To a suspension of the nucleoside (1.0 mol equivalent) in dry tetrahydrofuran, triethylamine (1.0 mol equivalent) was added. After stirring for 30 min at ambient temperature, phosphorus oxychloride (1.0 mol equivalent) was added dropwise at -78 °C. The reaction mixture was stirred for 30 min at -78 °C then allowed to warm to ambient temperature and stirred for additional 30 min. Reaction progress was monitored by ^{31}P NMR. After reaction completion, dry dichloromethane was added, followed by amino acid ester or amine (1 mol equivalent) and triethylamine (2 or 1 mol equivalent, respectively) at -78 °C. Reaction was warmed to ambient temperature and monitored by ^{31}P NMR. When NMR indicated completion of the reaction (no starting material, presence of mono-substituted product) 2nd amino acid ester or amine (5 mol equivalent) was added followed by the addition of triethylamine (10 or 5 mol equivalent, respectively) at -78°C. After stirring at ambient temperature for 16-20 h, reaction mixture was evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as eluent a gradient of methanol in chloroform.

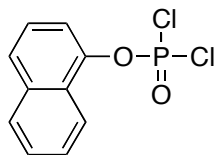
Standard procedure 13c: Preparation of asymmetrical phosphorodiamidates (amino acid dichloridate route).

To a suspension of the nucleoside (1.0 mol equivalent) in dry tetrahydrofuran, triethylamine (1.0 equivalent) was added. After stirring for 30 min at ambient temperature amino acid ester dichloridate (2.0 mol equivalent) was added. After stirring at ambient temperature for 20 h, primary amine was added (5.0 mol

equivalent) followed by the addition of triethylamine (5.0 mol equivalent) at -78 °C. After stirring at ambient temperature for 16-20 h, the reaction mixture was evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as eluent a gradient of methanol in chloroform.

Standard procedure 14: C6-Methyl ether cleavage.

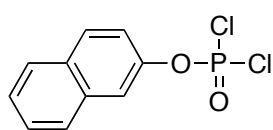
To a solution of appropriate ProTide (1 mol equivalent) in dry ACN, sodium iodide (1.1 mol equivalent), followed by trimethylsilyl chloride (1.1 mol equivalent) was added. Reaction mixture was stirred at ambient temperature (unless otherwise stated) and monitored by TLC. After reaction completion, the reaction mixture was poured into saturated solution of sodium bicarbonate and extracted 3 times with dichloromethane. The organic layers were combined, dried over Na₂SO₄ and evaporated to dryness. Crude mixture was purified using preparative TLC, with CHCl₃/MeOH as an eluent.

12.3 Experimental section - Chapter Three.**Synthesis of α -naphthyl phosphorodichloridate (3.1a).**

Prepared according to the Standard Procedure 1, from: α -naphthol (5.00g, 34.7 mmol), phosphorus oxychloride (3.23 ml, 34.7 mmol) and triethylamine (4.84 ml, 34.7 mmol) in 25 ml of dry diethyl ether. Product was obtained as thick, yellow oil (8.10g, 90%).

^{31}P NMR (202 MHz, CDCl_3) δ 3.69.

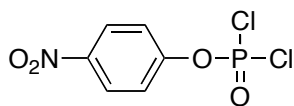
^1H NMR (500 MHz, CDCl_3) δ 8.11 (d, $J = 8.4$ Hz, 1H, H_8), 7.89 (d, $J = 7.5$ Hz, 1H, H_5), 7.81 (d, $J = 8.3$ Hz, 1H, H_4), 7.65 – 7.54 (m, 3H, H_2 , H_6 , H_7), 7.45 (t, $J = 8.0$ Hz, 1H, H_3).

Synthesis of β -naphthyl phosphorodichloridate (3.1b).

Prepared according to the Standard Procedure 1, from: β -naphthol (5.00g, 34.7 mmol), phosphorus oxychloride (3.23 ml, 34.7 mmol) and triethylamine (4.84 ml, 34.7 mmol) in 25 ml of dry diethyl ether. Product was obtained as thick, yellow oil (7.95g, 88%).

^{31}P NMR (202 MHz, CDCl_3) δ 2.71.

^1H NMR (500 MHz, CDCl_3) δ 7.87- 7.78 (m, 2H, H_4 , H_5 -naph), 7.72 (s, 1H, H_8 -naph), 7.53- 7.45 (m, 2H, H_7 , H_6 -naph), 7.39- 7.35 (m, 2H, H_1 , H_3 -naph).

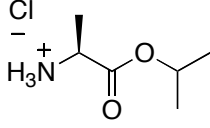
Synthesis of p -NO₂-phenyl phosphorodichloridate (3.1c).

Prepared according to the Standard Procedure 1, from: p -NO₂-phenol (1.60g, 11.50 mmol), phosphorus oxychloride (1.07 ml, 11.50 mmol) and triethylamine (1.60 ml, 11.50 mmol) in 10 ml of dry diethyl ether. Product was obtained as thick, yellow oil (2.54, 86%).

^{31}P NMR (202 MHz, CDCl_3) δ 3.54.

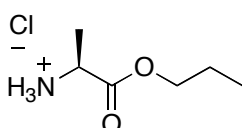
^1H NMR (500 MHz, CDCl_3) δ 8.35 (d, $J=9.00$ Hz, 2H, H_3 and H_5 Ph), 7.54-7.50 (m, 2H, H_2 and H_6 Ph).

Synthesis of L-alanine isopropyl ester hydrochloride salt (3.2a).


 Prepared according to the Standard Procedure 2a, from: L-alanine (5.00g, 56.0 mmol), thionyl chloride (8.20ml, 112.0 mmol) in 65 ml of isopropanol. The product was obtained as a white solid (8.0g, 85%).

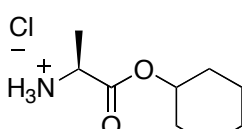
^1H NMR (500 MHz, MeOD) δ 5.18- 5.04 (m, 1H, $\text{CH}(\text{CH}_3)_2$), 4.05 (q, $J=7.5$ Hz, 1H, CHCH_3), 1.56 (d, $J=7.5$ Hz, 3H, CHCH_3), 1.36, 1.31 (2d, $J=6.5$ Hz, 6H, $\text{CH}(\text{CH}_3)_2$).

Synthesis of L-alanine n-propyl ester hydrochloride salt (3.2b).


 Prepared according to the standard procedure 2a, from: L-alanine (3.00g, 33.67 mmol), thionyl chloride (4.91ml, 67.00 mmol) and n-propanol (40 ml, 0.51 mol). The product was obtained as a white solid (4.90g, 87%).

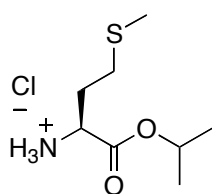
^1H NMR (500 MHz, CDCl_3) δ 8.85 (bs, 3H, NH_3^+), 4.26- 4.18 (m, 3H, $\text{OCH}_2\text{CH}_2\text{CH}_3$ and CHCH_3), 1.75- 1.71 (m, 5H, CHCH_3 and $\text{OCH}_2\text{CH}_2\text{CH}_3$), 0.97 (t, $J=4.6$ Hz, 3H, $\text{OCH}_2\text{CH}_2\text{CH}_3$).

Synthesis of L-alanine cyclohexyl ester hydrochloride salt (3.2c).


 Prepared according to the standard procedure 2a, from L-alanine (5.00g, 56.0 mmol) thionyl chloride (8.20 ml, 5.60 mmol) and cyclohexanol (90g, 0.90 mol). Product was obtained as a white solid (12.2g, 95%).

^1H NMR (500 MHz, CDCl_3) δ 8.76 (bs, 3H, NH_3), 4.91–4.79 (m, 1H, CH ester), 4.22 (q, $J = 7.5$ Hz, 1H, CHCH_3), 1.86–1.73 (m, 7H, 2x CH_2 ester and CHCH_3), 1.56–1.46 (m, 4H, 2x CH_2 ester), 1.42 – 1.23 (m, 2H, CH_2 ester).

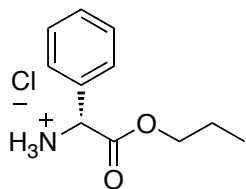
Synthesis of L-methionine isopropyl ester hydrochloride salt (3.2d).



Prepared according to the Standard Procedure 2a, from: L-methionine (5.00g, 33.51 mmol), thionyl chloride (4.9 ml, 67.01 mmol) and isopropanol (40 ml, 0.50 mol). Product was obtained as a white solid (7.61g, 98%).

^1H NMR (500 MHz, DMSO) δ 8.54 (s, 3H, NH_3^+), 5.06–4.98 (m, 1H, $\text{OCH}(\text{CH}_3)_2$), 4.05 (t, $J = 6.0$ Hz, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.66 (dt, $J = 7.5$ Hz, $J = 15.0$ Hz, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.54 (dt, $J = 7.5$ Hz, $J = 15.0$, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.09–2.03 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.07 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.27 (d, $J = 3.5$ Hz, 3H, $\text{OCH}(\text{CH}_3)_2$), 1.25 (d, $J = 3.5$ Hz, 3H, $\text{OCH}(\text{CH}_3)_2$).

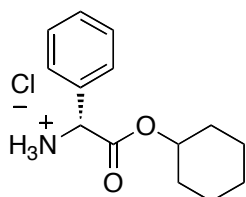
Synthesis of D-phenylglycine propyl ester hydrochloride salt (3.2e).



Prepared according to the Standard Procedure 2a, from: D-phenylglycine (3.00g, 19.84 mmol), thionyl chloride (2.88 ml, 39.69 mmol) and *n*-propanol (22 ml, 0.30 mol). Product was obtained as a white solid (4.54, 100%).

^1H NMR (500 MHz, DMSO) δ 9.16 (s, 3H, NH_3^+), 7.54–7.56 (m, 5H, CHPh), 5.23 (s, 1H, CHPh), 4.11 (t, $J = 7.5$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.56–1.49 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 0.74 (t, $J = 7.5$ Hz, 3H, $\text{OCH}_2\text{CH}_2\text{CH}_3$).

Synthesis of D-phenylglycine cyclohexyl ester hydrochloride salt (3.2f).

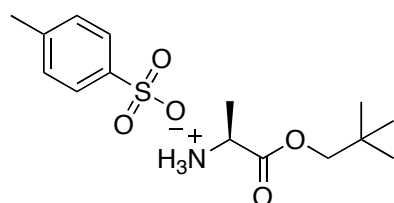


Prepared according to the Standard Procedure 2a, from: D-phenylglycine (5.00g, 33.08 mmol), thionyl chloride (4.90 ml, 66.15 mmol) and cyclohexanol (52 ml, 0.50 mol). Product

was obtained as a white solid (8.85, 99%).

^1H NMR (500 MHz, DMSO) δ 9.17 (s, 3H, NH_3^+), 7.53-7.45 (m, 5H, CHPh), 5.18 (s, 1H, CHPh), 4.81-4.79 (m, 1H, OCH ester), 1.77-1.72 (m, 1H, CH_2 ester), 1.60-1.46 (m, 1H, CH_2 ester), 1.32-1.24 (m, 4H, 2x CH_2 ester), 1.22-1.16 (m, 4H, 2x CH_2 ester).

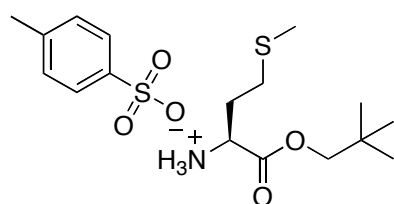
Synthesis of L-alanine 2,2-dimethylpropyl ester *p*-toluene sulfonate salt (3.2g).



Prepared according to the standard procedure 2b, from: L-alanine (8.00g, 89.8 mmol) neopentyl alcohol (40g, 45.4 mol), *p*TSA (18.96g, 98.9 mmol) in 120 ml of toluene. The product was obtained as a white powder (28g, 97%).

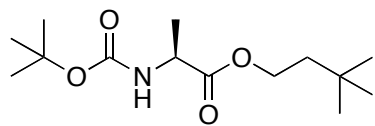
^1H NMR (500 MHz, DMSO) δ 8.29 (bs, 3H, NH_3^+), 7.49 (d, J = 8.0 Hz, 2H, Ts), 7.12 (d, J = 8.0, 2H, Ts), 4.16 (q, J = 7.2 Hz, 1H, CHCH_3), 3.95, 3.82 (AB, J_{AB} = 10.5 Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.30 (s, 3H, CH_3 Ts), 1.43 (d, J = 7.2 Hz, 3H, CHCH_3), 0.94 (s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of L-methionine 2,2-dimethylpropyl ester *p*-toluene sulfonate salt (3.2h).



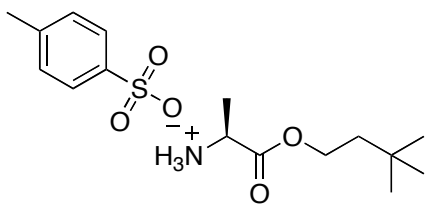
Prepared according to the standard procedure 2b, from: L-methionine (3.0g, 20.00 mmol), neopentyl alcohol (17.63g, 0.20 mol) and *p*TSA (9.48g, 22.00 mmol) in 60ml of toluene. The product was obtained as a white powder (7.20g, 91%).

^1H NMR (500 MHz, MeOD) δ 7.73 (d, J = 8.5 Hz, 2H, Ts), 7.26 (d, J = 8.5 Hz, 2H, Ts), 4.27 (t, J = 6.5 Hz, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 3.98, 3.85 (AB, J_{AB} = 10.5 Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.69, 2.68 (2x t, J = 7.0 Hz, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.39 (s, 3H, CH_3 Ts), 2.30-2.14 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.13 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.00 (s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of Boc-L-alanine 3,3-dimethyl-1-butyl ester (3.2i').

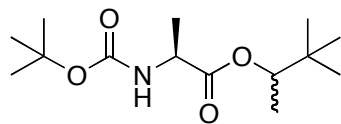
Prepared according to the standard procedure 2c, from: Boc-L-alanine (5.00g, 26.43 mmol), 3,3-dimethyl-1-butanol (5.00 ml, 39.64 mmol), DCC (5.45g, 26.43 mmol), DMAP (0.32g, 2.64 mmol) in 100ml of DCM. The product was obtained as clear oil (6.32g, 87%).

^1H NMR (500 MHz, CDCl_3) δ 5.06 (bs, 1H, NH), 4.34- 4.27 (m, 1H, CHCH_3), 4.23- 4.18 (m, 2H, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$), 1.59 (t, $J= 7.0$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$), 1.46 (s, 9H, 3x CH_3 Boc), 1.39 (d, $J= 7.0$ Hz, 3H, CHCH_3), 0.96 (s, 9H, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of L-alanine 3,3-dimethyl-1-butyl ester *p*-toluene sulfonate salt (3.2i).

Prepared according to the standard procedure 2d, from: Boc-L-alanine 3,3-dimethyl-1-butyl ester (6.32g, 23.12 mmol) and *p*TSA (4.40g, 23.12 mmol) in 220 ml of AcOEt. The product was obtained as a white solid (5.35g, 67%).

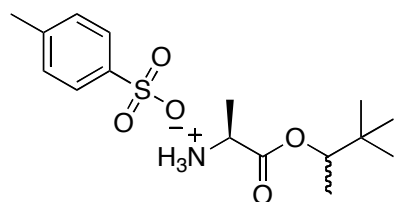
^1H NMR (500 MHz, CDCl_3) δ 8.22 (bs, 3H, NH_3^+), 7.79 (d, $J= 9.3$ Hz, 2H, Ts), 7.17 (d, $J= 9.3$ Hz, 2H, Ts), 4.19- 4.06 (m, 2H, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$), 3.99-3.94 (m, 1H, CHCH_3), 2.37 (s, 3H, CH_3 Ts), 1.52-1.44 (m, 5H, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$ and CHCH_3), 0.90 (s, 9H, s, $\text{OCH}_2\text{CH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of Boc-L-alanine 3,3-dimethyl-2-butyl ester (3.2j').

Prepared according to the standard procedure 2c, from: Boc-L-alanine (5.00g, 26.43 mmol), 3,3-dimethyl-2-butanol (4.32 ml, 34.35 mmol), DCC (5.45g, 26.43 mmol), DMAP (0.32g, 2.64 mmol) in 100ml of DCM. The product was obtained as clear oil (6.53g, 90%).

^1H NMR (500 MHz, CDCl_3) δ 5.09 (bs, 1H, NH), 4.75-4.70 (m, 1H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 4.33-4.25 (m, 1H, CHCH_3), 1.45, 1.44 (2x s, 3x CH_3 Boc), 1.41-1.36 (m, 3H, CHCH_3), 1.17- 1.13 (m, 3H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 0.92, 0.91 (2x s, 9H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$).

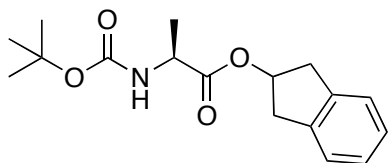
Synthesis of L-alanine 3,3-dimethyl-2-butyl ester *p*-toluene sulfonate salt (3.2j).



Prepared according to the standard procedure 2d, from: Boc-L-alanine 3,3-dimethyl-2-butyl ester (6.53g, 23.89 mmol) and *p*TSA (4.54g, 23.89 mmol) in 230 ml of AcOEt. The product was obtained as a white solid (5.52g, 67%).

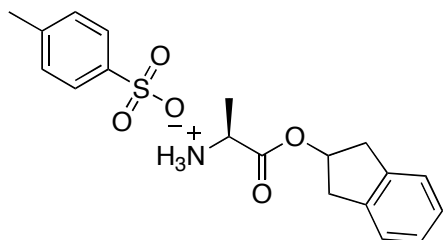
^1H NMR (500 MHz, CDCl_3) δ 8.23 (bs, 3H, NH_3^+), 7.79 (d, J = 8.0 Hz, 2H, Ts), 7.15 (d, J = 8.0 Hz, 2H, Ts), 4.71, 4.70 (2x q, J = 6.5 Hz, 1H, CHCH_3), 4.03-3.96 (m, 1H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 2.36 (s, 1H, CH_3 Ts), 1.48, 1.46 (2x d, J = 6.5 Hz, 3H, CHCH_3), 1.10, 1.08 (2x d, J = 6.5 Hz, 3H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 0.87, 0.85 (2x s, 9H, $\text{CH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$).

Synthesis of Boc-L-alanine 2-indanol ester (3.2k').



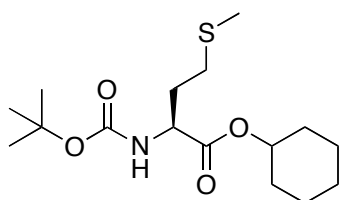
Prepared according to the standard procedure 2c, from: Boc-L-alanine (5.00g, 26.43 mmol), 2-indanol (5.32g, 39.64 mmol), DCC (5.45g, 26.43 mmol), DMAP (0.32g, 2.64 mmol) in 100ml of DCM. The product was obtained as clear oil (7.20g, 89%).

^1H NMR (500 MHz, CDCl_3) δ 7.24- 7.20 (m, 4H, Ar), 5.63- 5.59 (m, 1H, OCH ester), 5.03 (bs, 1H, NH), 4.28-4.26 (m, 1H, CHCH_3), 3.38 (dd, J = 7.00 Hz, J = 17.0 Hz, 2H, 2x CH_A ester), 3.07 (d, J = 17.0 Hz, 2H, 2 x CH_{B1} ester), 3.01 (d, J = 17.0 Hz, 2H, 2 x CH_{B2} ester), 1.45 (s, 9H, 3x CH_3 Boc), 1.36 (d, J = 7.0 Hz, 3H, CHCH_3).

Synthesis of L-alanine 2-indanyl ester *p*-toluene sulfonate salt (3.2k).

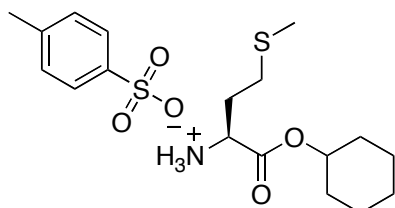
Prepared according to the standard procedure 2d, from: Boc-L-alanine 2-indanyl ester (7.20g, 23.58 mmol) and *p*TSA (4.50g, 23.58 mmol) in 250 ml of AcOEt. The product was obtained as a white solid (6.97g, 78%).

^1H NMR (500 MHz, CDCl_3) δ 8.21 (bs, 3H, NH_3^+), 7.76 (d, 2H, 2x CH Ts), 7.17-7.11 (m, 6H, 2x CH Tos, 4x CH Ar), 5.52-5.48 (m, 1H, CH ester), 3.96- 3.91 (m, 1H, CHCH_3), 3.25, 3.20 (2x dd, $J= 7.00$ Hz, $J= 17.0$ Hz, 2H, 2x CH_A ester), 2.96 (d, $J= 17.0$ Hz, 2H, 2x CH_B ester), 3.01 (2x d, $J= 17.0$ Hz, 2H, 2 x CH_B ester), 2.34 (s, 3H, CH_3 Tos), 1.40 (d, $J= 7.0$ Hz, 3H, CH_3).

Synthesis of Boc-L-methionine cyclohexyl ester (3.2l').

Prepared according to the standard procedure 2c, from: Boc-L-methionine (5.00g, 20.05 mmol), cyclohexanol (2.70 ml, 26.07 mmol), DCC (4.14g, 20.05 mmol), DMAP (0.24g, 2.01 mmol) in 100ml of DCM. The product was obtained as clear oil (5.90g, 87%).

^1H NMR (500 MHz, CDCl_3) δ 5.15 (bs, 1H, NH), 4.81-4.72 (m, 1H, OCH ester), 4.47-4.41 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.56-2.49 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.20-2.12 (m, 1H, 1 of $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.08 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.99- 1.92 (m, 1H, 1 of $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.77-1.72 (m, 1H, CH_2 ester), 1.62-1.48 (m, 1H, CH_2 ester), 1.43 (s, 9H, 3x CH_3 Boc), 1.32-1.24 (m, 4H, 2x CH_2 ester), 1.22-1.16 (m, 4H, 2x CH_2 ester).

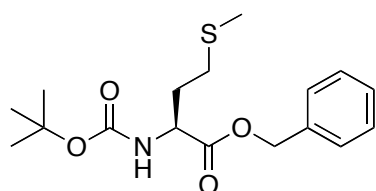
Synthesis of L-methionine cyclohexyl ester *p*-toluene sulfonate salt (3.2l).

Prepared according to the standard procedure 2d, from: Boc-L-methionine cyclohexyl ester (5.90g,

17.38 mmol) and *p*TSA (3.31g, 17.38 mmol) in 205 ml of AcOEt. The product was obtained as a white solid (5.50g, 77%).

^1H NMR (500 MHz, CDCl_3) δ 8.37 (bs, 3H, NH_3^+), 7.77 (d, J = 8.5 Hz, 2H, Ts), 7.13 (d, J = 8.5 Hz, 2H, Ts), 4.83-4.77 (m, 1H, OCH ester), 4.12-4.02 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.61-2.57, 2.54-2.49 (2m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.34 (s, 3H, CH_3 Ts), 2.18-2.09 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.98 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.81-1.72 (m, 2H, CH_2 ester), 1.69-1.61 (m, 2H, CH_2 ester), 1.42-1.21 (m, 6H, 3x CH_2 ester).

Synthesis of Boc-L-methionine benzyl ester (3.2m').

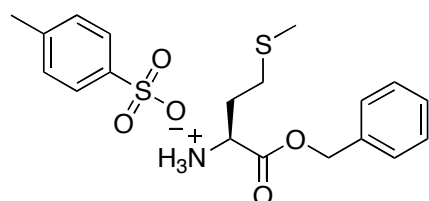


Prepared according to the standard procedure 2c, from: Boc-L-methionine (5.00g, 20.05 mmol), benzyl alcohol (2.70 ml, 26.07 mmol), DCC (4.14g, 20.05 mmol), DMAP (0.24g, 2.01 mmol) in 100ml of DCM.

The product was obtained as clear oil (5.90g, 87%).

^1H NMR (500 MHz, CDCl_3) δ 7.39-7.35 (m, 5H, OCH_2Ph), 5.23, 5.17 (AB, J_{AB} = 12.0 Hz, 2H, OCH_2Ph), 5.15 (bs, 1H, NH), 4.47- 4.41 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.52-2.49 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.20-2.12 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.06 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.99- 1.92 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.90 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.45 (s, 9H, 3x CH_3 Boc).

Synthesis of L-methionine benzyl ester *p*-toluene sulfonate salt (3.2m).



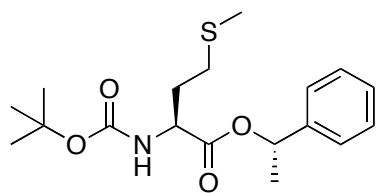
Prepared according to the standard procedure 2d, from: Boc-L-methionine benzyl ester (5.90g, 17.38 mmol) and *p*TSA (3.31g, 17.38 mmol) in 205 ml of AcOEt. The product was obtained as a

white solid (5.50g, 77%).

^1H NMR (500 MHz, CDCl_3) δ 8.39 (bs, 3H, NH_3^+), 7.77 (d, J = 8.5 Hz, 2H, Ts), 7.32-7.28 (m, 5H, OCH_2Ph), 7.12 (d, J = 8.5 Hz, 2H, Ts), 5.16, 5.04 (AB, J_{AB} = 12.5 Hz,

2H, OCH₂Ph), 4.20- 4.17 (m, 1H, CHCH₂CH₂SCH₃), 2.55-2.52 (dt, J = 7.0 Hz, J = 14.5 Hz, 1H, CHCH₂CH₂SCH₃), 2.43 (dt, J = 8.0 Hz, J = 14.5 Hz, 1H, CHCH₂CH₂SCH₃), 2.34 (s, 3H, CH₃ Ts), 2.21- 2.12 (m, 2H, CHCH₂CH₂SCH₃), 1.87 (s, 3H, CHCH₂CH₂SCH₃).

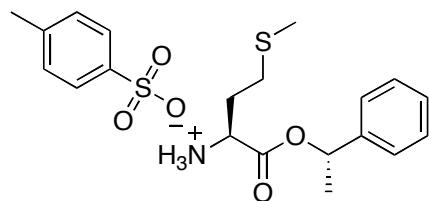
Synthesis of Boc-L-methionine *S*-phenylethyl ester (3.2n').



Prepared according to the standard procedure 2c, from: Boc-L-methionine (5.00 g, 20.05 mmol), *S*-phenylethanol (3.15 ml, 26.07 mmol), DCC (4.14 g, 20.05 mmol), DMAP (0.24g, 2.01 mmol) in 100 ml of DCM. The product was obtained as clear oil (6.50 g, 92%).

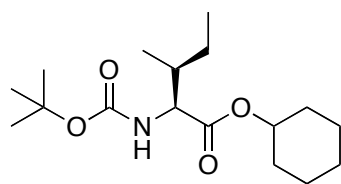
¹H NMR (500 MHz, CDCl₃) δ 7.37- 7.30 (m, 5H, OCH(CH₃)Ph), 5.94 (q, J = 7.0 Hz, 1H, OCH(CH₃)Ph), 4.47- 4.41 (m, 1H, CHCH₂CH₂SCH₃), 2.47- 2.42, 2.38- 2.32 (2x m, 2H, CHCH₂CH₂SCH₃), 2.11-2.06 (m, 1H, CHCH₂CH₂SCH₃), 2.01 (s, 3H, CHCH₂CH₂SCH₃), 1.93- 1.85 (m, 1H, CHCH₂CH₂SCH₃), 1.59 (d, J = 7.00 Hz, 3H, OCH(CH₃)Ph), 1.00 (s, 9H, 3x CH₃ Boc).

Synthesis of L-methionine *S*-phenylethyl ester *p*-toluene sulfonate salt (3.2n).



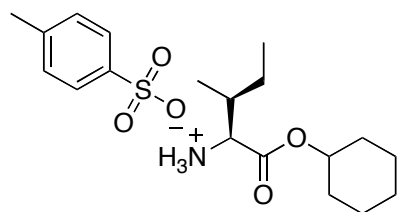
Prepared according to the standard procedure 2d, from: Boc-L-methionine *S*-phenylethyl ester (6.50 g, 18.49 mmol) and *p*TSA (3.52 g, 18.49 mmol) in 230 ml of AcOEt. The product was obtained as a white solid (4.67 g, 59%).

¹H NMR (500 MHz, CDCl₃) δ 8.34 (bs, 3H, NH₃⁺), 7.75 (d, J = 8.0 Hz, 2H, Ts), 7.33- 7.32 (m, 5H, OCH(CH₃)Ph), 7.12 (d, J = 8.0 Hz, 2H, Ts), 5.90 (q, J = 6.5 Hz, 1H, OCH(CH₃)Ph), 4.20- 4.16 (m, 1H, CHCH₂CH₂SCH₃), 2.45 (dt, J = 8.0 Hz, J = 14.5 Hz, 1H, CHCH₂CH₂SCH₃), 2.35 (s, 3H, CH₃ Ts), 2.30 (dt, J = 7.0 Hz, J = 14.5 Hz, 1H, CHCH₂CH₂SCH₃), 2.10- 2.06 (m, 2H, CHCH₂CH₂SCH₃), 1.83 (s, 3H, CHCH₂CH₂SCH₃), 1.50 (d, J = 6.5 Hz, 3H, OCH(CH₃)Ph).

Synthesis of Boc-L-isoleucine cyclohexyl ester (3.2o').

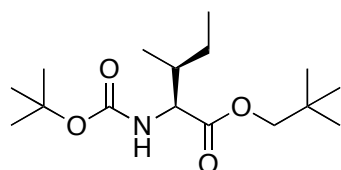
Prepared according to the standard procedure 2c, from: Boc-L-isoleucine (5.00g, 21.62 mmol), cyclohexanol (3.38 ml, 32.43 mmol), DCC (4.46 g, 21.62 mmol), DMAP (0.26 g, 2.16 mmol) in 100 ml of DCM. The product was obtained as clear oil (7.20 g, 100%).

^1H NMR (500 MHz, CDCl_3) δ 5.07 (bs, 1H, NH), 4.82-4.74 (m, 1H, OCH ester), 4.27-4.22 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 1.91-1.12 (m, 3H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ and CH_2 ester), 1.77-1.72 (m, 2H, CH_2 ester), 1.48 (s, 9H, 3x CH_3 Boc), 1.47-1.17 (m, 8H, 3x CH_2 ester and $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.99-0.87 (m, 6H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ and $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$).

Synthesis of L-isoleucine cyclohexyl ester *p*-toluene sulfonate salt (3.2o).

Prepared according to the standard procedure 2d, from: Boc-L-isoleucine cyclohexyl ester (7.20 g, 21.62 mmol) and *p*TSA (4.37 g, 21.62 mmol) in 250 ml of AcOEt. The product was obtained as a white solid (4.70 g, 53%).

^1H NMR (500 MHz, CDCl_3) δ 8.17 (bs, 1H, NH_3^+), 7.78 (d, J = 8.0 Hz, 2H, Ts), 7.13 (d, J = 8.0 Hz, 2H, Ts), 4.84-4.77 (m, 1H, OCH ester), 3.98-3.91 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 2.35 (s, 3H, CH_3 Ts), 2.01-1.97 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 1.87-1.85 (m, 2H, CH_2 ester), 1.77-1.72 (m, 2H, CH_2 ester), 1.456-1.21 (m, 8H, 3x CH_2 ester and $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.92 (d, J = 6.5 Hz, 3H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.73 (t, J = 7.5 Hz, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$).

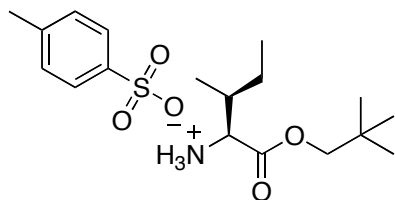
Synthesis of Boc-L-isoleucine 2,2-dimethylpropyl ester (3.2p').

Prepared according to the standard procedure 2c, from: Boc-L-isoleucine (5.00g, 21.62 mmol), 2,2-

dimethylpropanol (2.68 ml, 32.43 mmol), DCC (4.46 g, 21.62 mmol), DMAP (0.26 g, 2.16 mmol) in 100 ml of DCM. The product was obtained as clear oil (7.14 g, 100%).

^1H NMR (500 MHz, CDCl_3) δ 5.06 (bs, 1H, NH), 4.43-4.37 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 3.76, 3.62 (AB, $J_{\text{AB}}=12.0$ Hz, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.91-1.89 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 1.49 (s, 9H, 3x CH_3 Boc), 1.27-1.18 (m, 2H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.99-0.87 (m, 6H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$ and $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$).

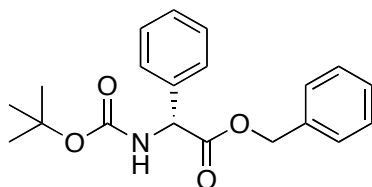
Synthesis of L-isoleucine 2,2-dimethylpropyl ester *p*-toluene sulfonate salt (3.2p).



Prepared according to the standard procedure 2d, from: Boc-L-isoleucine 2,2-dimethylpropyl ester (7.14 g, 23.68 mmol) and *p*TSA (4.51 g, 23.68 mmol) in 250 ml of AcOEt. The product was obtained as a white solid (4.10 g, 46%).

^1H NMR (500 MHz, CDCl_3) δ 8.21 (bs, 1H, NH_3^+), 7.79 (d, $J=8.0$ Hz, 2H, Ts), 7.13 (d, $J=8.0$ Hz, 2H, Ts), 4.08-3.99 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 3.81, 3.77 (AB, $J_{\text{AB}}=12.0$ Hz, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.34 (s, 3H, CH_3 Ts), 2.04-1.98 (m, 1H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 1.46-1.41, 1.32-1.79 (2m, 2H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.92 (d, $J=6.5$ Hz, 3H, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$), 0.90 (s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 0.74 (t, $J=7.5$ Hz, $\text{CHCH}(\text{CH}_3)\text{CH}_2\text{CH}_3$).

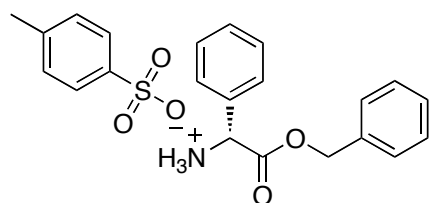
Synthesis of Boc-D-phenylglycine benzyl ester (3.2r').



Prepared according to the standard procedure 2c, from: Boc-D-phenylglycine (1.5 g, 0.74 mmol), benzyl alcohol (0.97 ml, 0.96 mmol), DCC (1.49 g, 0.74 mmol), DMAP (0.09 g, 0.07 mmol) in 35 ml of DCM. The product was obtained as clear oil (2.10 g, 98%).

^1H NMR (500 MHz, CDCl_3) δ 7.38-7.21 (m, 10H, CHPh and OCH_2Ph), 5.58 (bs, 1H, NH), 5.41 (bs, 1H, CHPh), 5.19 (s, 2H, OCH_2Ph), 1.46 (s, 9H, 3x CH_3 Boc).

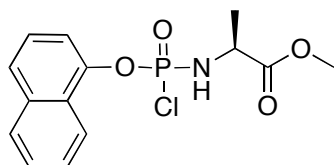
Synthesis of D-phenylglycine benzyl ester *p*-toluene sulfonate salt (3.2r).



Prepared according to the standard procedure 2d, from: Boc-D-phenylglycine benzyl ester (2.10 g, 0.71 mmol) and *p*TSA (1.34 g, 0.71 mmol) in 75 ml of AcOEt. The product was obtained as a white solid (1.78 g, 61%).

^1H NMR (500 MHz, CDCl_3) δ 8.22 (bs, 1H, NH_3^+), 7.50-7.47 (m, 7H, CHPh and OCH_2Ph), 7.36-7.33 (m, 3H, OCH_2Ph and Ts), 7.25-7.23 (m, 2H, OCH_2Ph), 7.11 (d, $J = 7.5$ Hz, 2H, Ts), 5.40 (s, 1H, CHPh), 5.28, 5.22 (AB, $J_{\text{AB}} = 12.5$ Hz, 2H, OCH_2Ph), 2.30 (s, 3H, Ts).

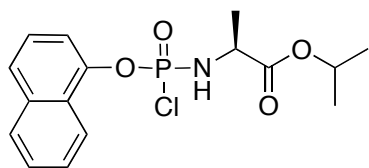
Synthesis of α -naphthyl-(methoxy-L-alaninyl) phosphorochloridate (3.3a).



Prepared to the Standard Procedure 3, from L-alanine methyl ester hydrochloride salt: (1.00g, 7.16 mmol), α -naphthyl phosphorodichloridate (1.87g, 7.16 mmol) and Et_3N (3.99 ml, 14.32 mmol) in 15 ml of dry DCM. Product was obtained as a pale, yellow, thick oil (1.21g, 52%).

^{31}P NMR (202 MHz, CDCl_3) δ 8.19, 7.92.

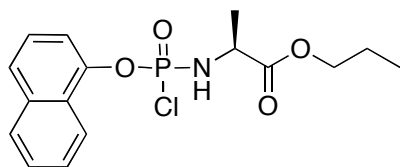
^1H NMR (500 MHz, CDCl_3) δ 8.12, 8.10 (2d, $J = 7.5$ Hz, 1H, $\text{H}_8\text{-Naph}$), 7.90 (d, $J = 7.5$ Hz, 1H, $\text{H}_5\text{-Naph}$), 7.76 (d, $J = 8.0$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.68 – 7.55 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.46 (t, $J = 8.0$ Hz, 1H, $\text{H}_3\text{-Naph}$), 4.52, 4.45 (2bs, 1H, NH), 4.36- 4.30 (m, 1H, CHCH_3), 3.83, 3.79 (2s, 3H, OCH_3), 1.59, 1.56 (2d, $J = 7.0$ Hz, 3H, CHCH_3).

Synthesis of α -naphthyl-(isopropoxy-L-alaninyl) phosphorochloridate (3.3b).

Prepared according to the Standard Procedure 3, from: α -naphthyl (2.70g, 10.33 mmol), L-alanine isopropyl ester hydrochloride salt (1.40g, 10.33 mmol), and Et₃N (2.88 ml, 20.65 mmol) in 15 ml of dry DCM. Product was obtained as a pale, yellow, thick oil (1.10g, 30%).

³¹P NMR (202 MHz, CDCl₃) δ 8.29, 7.97.

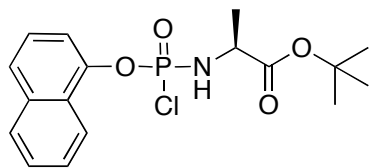
¹H NMR (500 MHz, CDCl₃) δ 8.12, 8.11 (2x d, J = 8.0 Hz, 1H, H₈-Naph), 7.91-7.89 (m, 1H, H₅-Naph), 7.75 (d, J = 8.5 Hz, 1H, H₄-Naph), 7.64–7.56 (m, 3H, H₇, H₆, H₂-Naph), 7.47, 7.46 (2t, J = 8.0 Hz, 1H, H₃-Naph), 5.17- 5.06 (m, 1H, OCH(CH₃)₂), 4.46- 4.31 (m, 1H NH), 4.30 – 4.23 (m, 1H, CHCH₃), 1.57, 1.55 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.33 (d, J = 6.0 Hz, 3H, OCH(CH₃)₂), 1.29 (d, J = 6.0, 3H, OCH(CH₃)₂).

Synthesis of α -naphthyl-(*n*-propoxy-L-alaninyl) phosphorochloridate (3.3c)

Prepared according to the Standard Procedure 3, from: L-alanine *n*-propyl ester hydrochloride salt (2.00g, 11.93 mmol), α -naphthyl phosphorodichloridate (3.11g, 11.93 mmol) and Et₃N (3.32 ml, 23.86 mmol) in 30 ml of dry DCM. The product was obtained as a clear, yellow, thick oil (3.50g, 82%).

³¹P NMR (202 MHz, CDCl₃) δ 8.29, 8.04

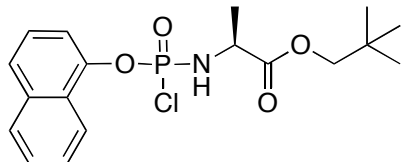
¹H NMR (500 MHz, CDCl₃) δ 8.13 – 8.04 (m, 1H, H₈-Naph), 7.89, 7.88 (2d, J = 7.5 Hz, 1H, H₅-Naph), 7.75 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64–7.55 (m, 3H, H₇, H₆, H₂-Naph), 7.45 (t, J = 8.0 Hz, 1H, H₃-Naph), 4.51, 4.25 (2bs, 1H, NH), 4.28–4.27 (m, 1H, CHCH₃), 4.15, 4.14 (2q, J = 7.0 Hz, 2H, OCH₂CH₂CH₃), 1.76-1.65 (m, 2H, OCH₂CH₂CH₃), 1.59, 1.56 (2d, J = 7.0 Hz, 3H, CHCH₃), 0.99, 0.96 (2t, J = 6.5 Hz, 3H, OCH₂CH₂CH₃).

Synthesis of α -naphthyl-(*t*-butoxy-L-alaninyl) phosphorochloridate (3.3d).

Prepared according to the Standard Procedure 3 from: L-alanine *t*-butyl ester hydrochloride salt (1.00g, 5.50 mmol), α -naphthyl phosphorodichloridate (1.43g, 5.50 mmol) and Et₃N (1.53 ml, 11.00 mmol) in 15 ml of dry DCM. Product was obtained as a pale, yellow, thick oil (1.48g, 69%).

³¹P NMR (202 MHz, CDCl₃) δ 8.43, 8.11.

¹H NMR (500 MHz, CDCl₃) δ 8.13, 8.10 (2d, J = 8.0 Hz, 1H, H₈-Naph), 7.89 (d, J = 8.3 Hz 1H, H₅-Naph), 7.75 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64–7.55 (m, 3H, H₇, H₆, H₂-Naph), 7.46 (t, J = 7.5 Hz, 1H, H₃-Naph), 4.53–4.41 (m, 1H NH), 4.23–4.16 (m, 1H, CHCH₃), 1.55, 1.53 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.53, 1.49 (2s, 9H, CH(CH₃)₃).

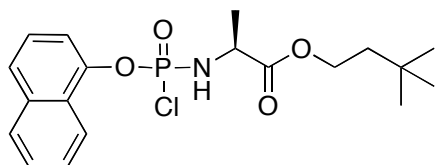
Synthesis of α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (3.3e).

Prepared according to the Standard Procedure 3, from: L-alanine 2,2-dimethylpropyl ester tosylate salt (5.00g, 15.09 mmol), α -naphthyl phosphorodichloridate (3.94g, 15.09 mmol) and Et₃N (4.20 ml, 30.17 mmol) in 66 ml of dry DCM. The product was obtained as clear, yellow, thick oil (5.70g, 99%).

³¹P NMR (202 MHz, CDCl₃) δ 8.21, 7.90.

¹H NMR (500 MHz, CDCl₃) δ 8.10 (d, J = 7.5 Hz 1H, H₈-Naph), 7.90–7.88 (m, 1H, H₅-Naph), 7.75 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64 – 7.55 (m, 3H, H₆, H₇, H₂-Naph), 7.45, 7.44 (2t, J = 8.0 Hz, 1H, H₃-Naph), 4.60–4.49 (m, 1H, NH), 4.40–4.30 (m, 1H, CHCH₃), 3.97, 3.93, 3.89, 3.83 (2AB, J_{AB} = 10.5 Hz 2H, OCH₂C(CH₃)₃), 1.61, 1.58 (2d, J = 7.0 Hz, 3H, CHCH₃), 0.99, 0.97 (2s, 9H, OCH₂C(CH₃)₃).

Synthesis of α -naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl) phosphorochloridate (3.3f).

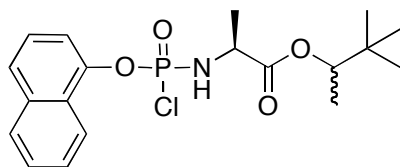


Prepared according to the Standard Procedure 3, from: L-alanine 3,3-dimethyl-1-butyl ester tosylate salt (2.00g, 5.79 mmol), α -naphthyl phosphorodichloridate (1.51g, 5.79 mmol) and Et₃N (1.61 ml, 11.58 mmol) in 30 ml of dry DCM. The product was obtained as clear, yellow, thick oil (2.10g, 91%).

³¹P NMR (202 MHz, CDCl₃) δ 8.46, 8.02.

¹H NMR (500 MHz, CDCl₃) δ 8.11, 8.09 (2d, J = 7.5 Hz 1H, H₈-Naph), 7.90-7.89 (m, 1H, H₅-Naph), 7.75 (d, J = 8.5 Hz, 1H, H₄-Naph), 7.64 – 7.55 (m, 3H, H₆, H₇, H₂-Naph), 7.46 (t, J = 7.5 Hz, 1H, H₃-Naph), 4.56- 4.45 (m, 1H, NH), 4.32- 4.26 (m, 2H, OCH₂CH₂C(CH₃)₃), 4.25– 4.21 (m, 1H, CHCH₃), 1.64- 1.60 (m, 2H, OCH₂CH₂C(CH₃)₃), 1.58, 1.55(2d, J = 7.0 Hz, CHCH₃), 0.98, 0.96 (2s, 9H, OCH₂CH₂C(CH₃)₃).

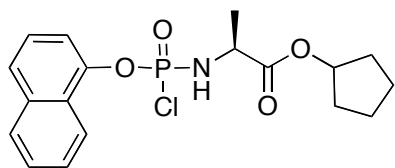
Synthesis of α -naphthyl-(3,3-dimethyl-2-butoxy-L-alaninyl) phosphorochloridate (3.3g).



Prepared according to the Standard Procedure 3, from: L-alanine 3,3-dimethyl-2-butyl ester tosylate salt (2.00g, 5.79 mmol), α -naphthyl phosphorodichloridate (1.51g, 5.79 mmol) and Et₃N (1.61 ml, 11.58 mmol) in 30 ml of dry DCM. The product was obtained as clear, yellow, thick oil (2.20g, 96%).

³¹P NMR (202 MHz, CDCl₃) δ 8.30, 8.24, 7.98, 7.95.

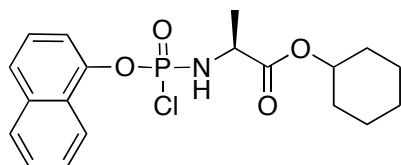
¹H NMR (500 MHz, CDCl₃) δ 8.12, 8.10 (2d, J = 6.5 Hz 1H, H₈-Naph), 7.91- 7.88 (m, 1H, H₅-Naph), 7.75 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64–7.55 (m, 3H, H₆, H₇, H₂-Naph), 7.46 (t, J = 8.0 Hz, 1H, H₃-Naph), 4.84- 4.77 (m, 1H, OCH(CH₃)C(CH₃)₃), 4.57- 4.42 (m, 1H, NH), 4.36– 4.25 (m, 1H, CHCH₃), 1.60, 1.58, 1.57, 1.56 (4d, J = 7.0 Hz, 3H, CHCH₃), 1.22, 1.20, 1.18, 1.17 (4d, J = 6.5 Hz, 3H, OCH(CH₃)C(CH₃)₃), 0.97, 0.96, 0.95, 0.93 (4s, 9H, OCH(CH₃)C(CH₃)₃).

Synthesis of α -naphthyl-(cyclopentoxo-L-alaninyl) phosphorochloridate (3.3h).

Prepared according to the Standard Procedure 3, from: L-alanine cyclopentyl ester tosylate salt (4.00g, 12.14 mmol), α -naphthyl phosphorodichloridate (3.17g, 12.14 mmol) and Et₃N (1.60 ml, 24.28 mmol) in 66 ml of dry DCM. Product was obtained as a yellow, clear, thick oil (3.01g, 65%).

³¹P NMR (202 MHz, CDCl₃) δ 8.38, 8.13.

¹H NMR (500 MHz, CDCl₃) δ 8.13- 8.09 (m, 1H, H₈-Naph), 7.95- 7.88 (m, 1H, H₅-Naph), 7.74 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64- 7.54 (m, 3H, H₇, H₆, H₂-Naph), 7.50- 7.40 (m, 1H, H₃-Naph), 5.30-5.28, 5.25-5.23 (2m, 1H, OCH ester), 4.63-4.57 (m, 1H, NH), 4.28-4.25 (m, 1H, CHCH₃), 1.94-1.85 (m, 2H, CH₂ ester), 1.79-1.69 (m, 4H, 2 x CH₂ ester), 1.66-1.59 (m, 2H, CH₂ ester), 1.57, 1.54 (2d, J = 7.0 Hz, 3H, CHCH₃).

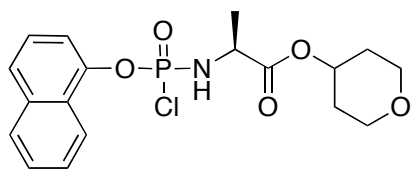
Synthesis of α -naphthyl-(cyclohexoxy-L-alaninyl) phosphorochloridate (3.3i).

Prepared according to the Standard Procedure 3, from: L-alanine cyclohexyl ester hydrochloride salt (5g, 24.07 mmol), α -naphthyl phosphorodichloridate (6.28g, 24.07 mmol) and Et₃N (3.35 ml, 48.14 mmol) in 100 ml of dry DCM. Product was obtained as yellow, clear, thick oil (8.72g, 92%).

³¹P NMR (202 MHz, CDCl₃) δ 8.29, 7.92.

¹H NMR (500 MHz, CDCl₃) δ 8.11, 8.10 (2d, J = 6.5 Hz, 1H, H₈-Naph), 7.91-7.89 (m, 1H, H₅-Naph), 7.76 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.64-7.55 (m, 3H, H₇, H₆, H₂-Naph), 7.47, 7.46 (2t, J = 8.0 Hz, 1H, H₃-Naph), 4.92-4.84 (m, 1H, OCH ester), 4.45-4.25 (m, 2H, NH and CHCH₃), 1.92-1.85 (m, 2H, CH₂ ester), 1.79-1.73 (m, 2H, CH₂ ester), 1.58, 1.56 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.61-1.31 (m, 6H, CH₂ ester).

Synthesis of α -naphthyl-(tetrahydropyranoxy-L-alanine) phosphorochloridate (3.3j).

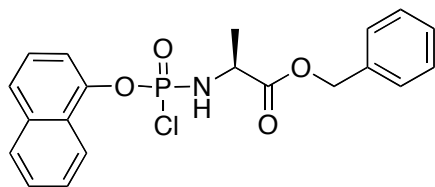


Prepared according to the standard procedure 3, from: L-alanine tetrahydropyranyl ester tosylate salt (1.00g, 2.90 mmol), α -naphthyl phosphorodichloridate (0.76g, 2.90 mmol) and Et₃N (0.80 ml, 5.79 mmol) in 30 ml of dry DCM. The product was obtained as yellow, thick oil in (0.84g, 70%).

³¹P NMR (202 MHz, CDCl₃) δ 8.19, 8.00.

¹H NMR (500 MHz, CDCl₃) δ 8.11, 8.08 (2d, J = 8.00 Hz, 1H, H₈-Naph), 7.91, 7.89 (2d, J = 8.50 Hz, 1H, H₅-Naph), 7.72, 7.66 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.61-7.51 (m, 3H, H₇, H₆, H₂), 7.47 (t, J = 8.00 Hz, 1H, H₃-Naph), 5.12-4.99 (m, 1H, OCH ester), 4.57 (bs, 1H, NH), 4.31-4.22 (m, 1H, CHCH₃), 3.98-3.80 (m, 2H, CH₂ ester), 3.53 – 3.45 (m, 2H, CH₂ ester), 1.97-1.88 (m, 2H, CH₂ ester), 1.71-1.62 (m, 2H, CH₂ ester), 1.54, 1.51 (2d, J = 7.00 Hz, 3H, CHCH₃).

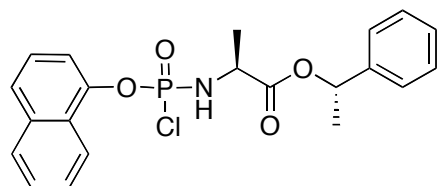
Synthesis of α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (3.3k).



Prepared according to the Standard Procedure 3, from L-alanine benzyl ester tosylate salt (10.00g, 28.46 mmol), α -naphthyl phosphorodichloridate (7.43g, 28.46 mmol) and Et₃N (7.92 ml, 56.91 mmol) in 200 ml of dry DCM). The product was obtained as clear, yellow, thick oil (9.80g, 85%).

³¹P NMR (202 MHz, CDCl₃) δ 8.16, 7.92.

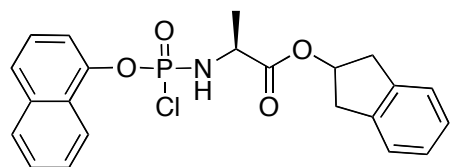
¹H NMR (500 MHz, CDCl₃) δ 8.10- 8.08 (m, 1H, H₈-Naph), 7.91- 7.88 (m, 1H, H₅-Naph), 7.75 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.60-7.58 (m, 3H, H₆, H₇, H₂-Naph), 7.45, 7.44 (2t, J = 8.0 Hz, 1H, H₃-Naph), 7.40-7.36 (m, 5H, OCH₂Ph), 5.26-5.17 (m, 2H, OCH₂Ph), 4.60-4.48, 4.53-4.49 (2m, 1H, NH), 4.41-4.34 (m, 1H, CHCH₃), 1.59, 1.57 (2d, J = 7.5 Hz, 3H, CHCH₃).

Synthesis of α -naphthyl-(*S*-phenylethoxy-L-alanine) phosphorochloridate (3.3l).

Prepared according to the Standard Procedure 3, from: L-alanine *S*-phenylethyl ester tosylate salt (7.70g, 21.07 mmol), α -naphthyl phosphorodichloridate (5.50g, 21.07 mmol), Et₃N (3.80 ml, 42.13 mmol) in 80 ml of rdy DCM. Product was obtained as yellow, thick oil (6.00g, 68%).

³¹P NMR (202 MHz, CDCl₃) δ 8.22, 7.94.

¹H NMR (500 MHz, CDCl₃) δ 8.10 (d, J = 7.5 Hz, 1H, H₈-Naph), 7.91- 7.89 (m, 1H, H₅-Naph), 7.75 (d, J = 8.5 Hz, 1H, H₄-Naph), 7.64-7.56 (m, 3H, H₇, H₆, H₂-Naph), 7.48 – 7.42 (m, 1H, H₃-Naph), 7.39-7.36 (m, 5H, OCH(CH₃)Ph), 5.98, 5.94 (2q, J = 7.0 Hz, 1H, OCH(CH₃)Ph), 4.48-4.41 (m, 1H, NH), 4.39-4.32 (m, 1H, CHCH₃), 1.63, 1.59 (2 d, J = 7.0 Hz, 3H, OCH(CH₃)Ph), 1.54, 1.51 (2d, J = 7.0 Hz, 3H, CHCH₃).

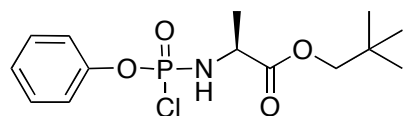
Synthesis of α -naphthyl-(2-indanoxy-L-alaninyl) phosphorochloridate (3.3m).

Prepared according to the Standard Procedure 3, from: L-alanine 2-indanyl ester tosylate salt (2.00g, 5.30 mmol), α -naphthyl phosphorodichloridate (1.38g, 5.30 mmol), Et₃N (1.48 ml, 10.60 mmol) in 15 ml of rdy DCM. Product was obtained as yellow, thick oil (1.48g, 65%).

³¹P NMR (202 MHz, CDCl₃) δ 8.16, 7.90.

¹H NMR (500 MHz, CDCl₃) δ 8.08-8.05 (m, 1H, H₈-Naph), 7.90-7.88 (m, 1H, H₅-Naph), 7.75 (d, J = 8.5 Hz, 1H, H₄-Naph), 7.62-.52 (m, 3H, H₇, H₆, H₂-Naph), 7.45, 7.44 (2t, J = 8.0 Hz, 1H, H₃-Naph), 7.29- 7.20 (m, 4H, 4x CH Ar ester), 5.68-5.65, 5.63-5.59 (2m, 1H, OCH ester), 4.51, 4.25 (2bs, 1H, NH), 4.31-4.22 (m, 1H, CHCH₃), 3.42- 3.32 (m, 2H, CH₂ ester), 3.11- 3.00 (m, 2H, CH₂ ester), 1.54, 1.52 (2d, J = 7.0 Hz, 3H, CHCH₃).

Synthesis of phenyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (3.3n).

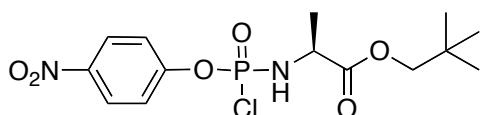


Prepared according to the Standard Procedure 3, from: L-alanine 2,2-dimethylpropyl ester tosylate salt (1.50g, 6.03 mmol), phenyl phosphorodichloridate (0.90 ml, 6.03 mmol) and Et₃N (1.68 ml, 12.06 mmol) in 66 ml of dry DCM. The product was obtained as clear, thick oil (1.91g, 95%).

³¹P NMR (202 MHz, CDCl₃) δ 8.02, 7.73.

¹H NMR (500 MHz, CDCl₃) δ 7.40- 7.23 (m, 5H, Ph), 4.53- 4.43 (m, 1H, NH), 4.30- 4.18 (m, 1H, CHCH₃), 3.94, 3.92, 3.88, 3.85 (2AB, *J*_{AB}= 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.56, 1.55 (2d, *J*= 5.5 Hz, 3H, CHCH₃), 0.98, 0.97 (2s, 9H, OCH₂C(CH₃)₃).

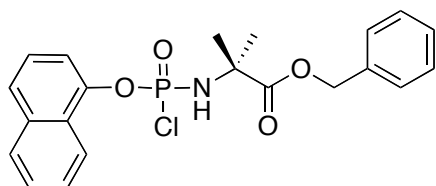
Synthesis of *p*-nitrophenyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (3.3o).



Prepared according to the Standard Procedure 3, from: L-alanine 2,2-dimethylpropyl ester tosylate salt (2.00g, 10.22 mmol), *p*-NO₂-phenyl phosphorodichloridate (2.62 g, 10.22 mmol) and Et₃N (2.85 ml, 20.43 mmol) in 66 ml of dry DCM. After reaction completion, solvent was removed under reduced pressure and residue was taken up in EtOAc and the resulting salts were filtered off. After evaporation, the product was obtained as clear, thick oil (3.2 g) and was used immediately for subsequent step without further purification.

³¹P NMR (202 MHz, CDCl₃) δ 8.10.

Synthesis of α-naphthyl-(benzoxy-dimethylglycynyl) phosphorochloridate (2.3p).



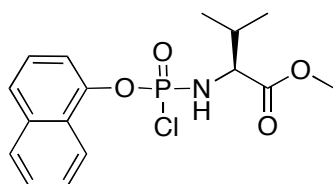
Prepared according to the Standard Procedure 3, from: dimethylglycine ester tosylate salt (2.00g,

6.03 mmol), α -naphthyl phosphorodichloridate (0.90 ml, 6.03 mmol) and Et₃N (1.68 ml, 12.06 mmol) in 66 ml of dry DCM. The product was obtained as clear, thick oil (1.91g, 95%).

³¹P NMR (202 MHz, CDCl₃) δ 5.86.

¹H NMR (500 MHz, CDCl₃) δ 8.13 (d, J = 7.0 Hz, 1H, H₈-Naph), 7.88 (d, J = 7.0 Hz 1H, H₅-Naph), 7.74 (d, J = 8.0 Hz, 1H, H₄-Naph), 7.58 – 7.55 (m, 3H, H₇, H₆, H₂-Naph), 7.44 (t, J = 8.0 Hz, 1H, H₃-Naph), 7.39 – 7.36 (m, 5H, OCH₂Ph), 5.25 (s, 2H, OCH₂Ph), 4.90 (bs, 1H, NH), 1.80 (s, 3H, C(CH₃)₂), 1.75 (s, 3H, C(CH₃)₂).

Synthesis of α -naphthyl-(methoxy-L-valinyl) phosphorochloride (3.3r).



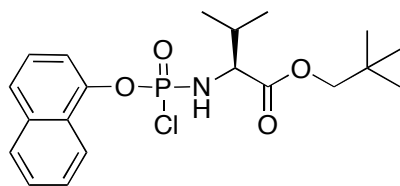
Prepared according to the standard procedure 3, from: L-valine methyl ester hydrochloride salt (2.00g, 11.93 mmol), α -naphthyl phosphorodichloridate (0.77g, 11.93 mmol) and Et₃N (4.11 ml, 23.85 mmol) in 20 ml of dry

DCM. Compound was obtained as a clear, yellow, thick oil (2.63g, 62%).

³¹P NMR (202 MHz, CDCl₃) δ 10.02, 9.56.

¹H NMR (500 MHz, CDCl₃) δ 8.12, 8.10 (2d, J = 7.50 Hz, 1H, H₈-Naph), 7.88 (d, J = 8.00 Hz, 1H, H₅-Naph), 7.74 (d, J = 8.00 Hz, 1H, H₄-Naph), 7.65-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.50, 7.45 (2t, J = 8.00 Hz, 1H, H₃-Naph), 4.59 (bs, 1H, NH), 4.09-4.06 (m, 1H, CHCH(CH₃)₂), 3.81, 3.76 (2s, 3H, OCH₃), 2.25 – 2.18 (m, 1H, CHCH(CH₃)₂), 1.06, 1.03 (2d, J = 6.50 Hz, 3H, CHCH(CH₃)₂), 0.99, 0.96 (2d, J = 6.50 Hz, 3H, CHCH(CH₃)₂).

Synthesis of α -naphthyl-(2,2-dimethylpropoxy-L-valinyl) phosphorochloride (3.3s).



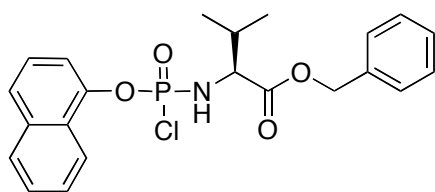
Prepared according to the Standard Procedure 3, from: L-valine neopentyl tosylate salt (2.00g, 5.56 mmol), α -naphthyl phosphorodichloridate (1.45g, 5.56 mmol) and Et₃N (1.55 ml, 11.13 mmol) in 32

ml of dry DCM. Product was obtained as pale, thick oil (1.75 g, 76%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.84, 9.34.

^1H NMR (500 MHz, CDCl_3) δ 8.13 – 8.11 (m, 1H, $\text{H}_8\text{-Naph}$), 7.90- 7.88 (m, 1H, $\text{H}_5\text{-Naph}$), 7.75 (d, $J = 8.5$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.60- 7.55 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.45 (t, $J = 8.0$ Hz, 1H, $\text{H}_3\text{-Naph}$), 4.42- 4.35 (m, 1H, NH), 4.17 – 4.11 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 3.94- 3.86 (m, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.28- 2.19 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.09, 1.06 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$), 1.02, 0.99 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$), 1.00, 0.98 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of α -naphthyl-(benzoxy-L-valinyl) phosphorochloridate (3.3t).



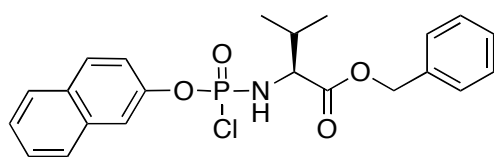
Prepared according to the Standard Procedure 3, from: L-valine benzyl ester tosylate salt (1.50 g, 3.95 mmol), α -naphthyl phosphorodichloridate (1.03g, 3.95 mmol), Et_3N (0.71 ml, 7.55 mmol)

in 30 ml of dry DCM. Product was obtained as yellow, thick oil (1.23g, 72%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.78, 9.29.

^1H NMR (500 MHz, CDCl_3) δ 8.12-8.10 (m, 1H, $\text{H}_8\text{-Naph}$), 7.90-7.88 (m, 1H, $\text{H}_5\text{-Naph}$), 7.76-7.74 (m, 1H, $\text{H}_4\text{-Naph}$), 7.65- 7.54 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.45 (t, $J = 8.0$ Hz, 1H, $\text{H}_3\text{-Naph}$), 7.42-7.32 (m, 5H, OCH_2Ph), 5.22-5.19 (m, 2H, OCH_2Ph), 4.17-4.11 (m, 2H, NH and $\text{CHCH}(\text{CH}_3)_2$), 2.28- 2.19 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.05, 1.02 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$), 1.01, 0.98 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$).

Synthesis of β -naphthyl-(benzoxy-L-valinyl) phosphorochloridate (3.3u).

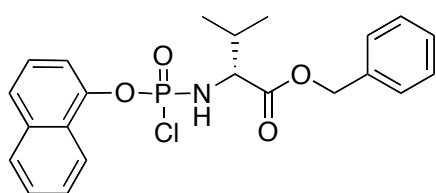


Prepared according to the Standard Procedure 3, from: L-valine benzyl ester tosylate salt (1.50 g, 3.95 mmol), β -naphthyl phosphorodichloridate (1.03g, 3.95 mmol), Et_3N (0.71 ml, 7.55 mmol) in 30 ml of dry DCM. Product was obtained as yellow, thick oil (1.69g, 99%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.71, 9.18.

^1H NMR (500 MHz, CDCl_3) δ 7.86–7.75 (m, 4H, H_4 , H_5 , H_8 , H_7 -Naph), 7.56–7.47 (m, 2H, H_6 , H_1 -Naph) 7.40–7.33 (m, 6H, H_3 -Naph and OCH_2Ph), 5.24–5.21 (m, 2H, OCH_2Ph), 4.64–4.59 (m, 1H, NH), 4.11–4.03 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 2.29–2.16 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.07, 1.04 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$), 0.97, 0.95 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$).

Synthesis of α -naphthyl-(benzoxy-D-valinyl) phosphorochloridate (3.3v).

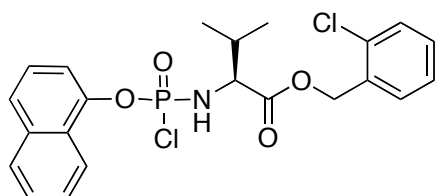


Prepared according to the Standard Procedure 3, from: D-valine benzyl ester tosylate salt (1.50 g, 3.95 mmol), α -naphthyl phosphorodichloridate (1.03g, 3.95 mmol), Et_3N (0.71 ml, 7.55 mmol) in 30 ml of dry DCM. Product was obtained as yellow, thick oil (1.70g, 99%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.71, 9.30

^1H NMR (500 MHz, CDCl_3) δ 8.11–8.08 (m, 1H, H_8 -Naph), 7.91–7.88 (m, 1H, H_5 -Naph), 7.75 (d, $J = 8.0$ Hz, 1H, H_4 -Naph), 7.64–7.54 (m, 3H, H_7 , H_6 , H_2 -Naph), 7.45 (t, $J = 7.5$ Hz, 1H, H_5 -Naph), 7.40–7.33 (m, 5H, OCH_2Ph), 5.23, 5.17 (AB, $J_{\text{AB}} = 11.5$ Hz, 2H, OCH_2Ph), 4.38–4.31 (m, 1H, NH), 4.17–4.10 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 2.26–2.18 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.05, 1.02 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$), 0.96, 0.93 (2d, $J = 7.0$ Hz, 3H, $\text{CHCH}(\text{CH}_3)_2$).

Synthesis of α -naphthyl-(*p*-chloro-benzoxy-L-valinyl) phosphorochloridate (3.3w).

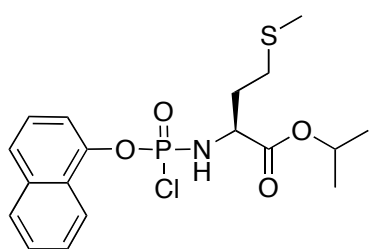


Prepared according to the Standard Procedure 3, from: L-valine *o*-chlorobenzyl ester tosylate salt (9.95g, 24.04 mmol) α -naphthyl phosphorodichloridate (6.27g, 24.04 mmol), Et_3N (6.77 ml, 48.07 mmol) in 135 ml of dry DCM. Product was obtained as yellow, thick oil (11.15g, 99%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.72, 9.24.

^1H NMR (500 MHz, CDCl_3) δ 8.09- 8.08 (m, 1H, $\text{H}_8\text{-Naph}$), 7.90-7.88 (m, 1H, $\text{H}_5\text{-Naph}$), 7.75 (d, $J = 8.5$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.58-7.30 (m, 8H, $\text{H}_7\text{-}$, $\text{H}_6\text{-}$, $\text{H}_3\text{-}$, $\text{H}_2\text{-Naph}$, and OCH_2Ph), 5.35- 5.25 (m, 2H, OCH_2Ph), 4.30- 4.22 (m, 1H, NH), 4.18- 4.11 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 2.27-2.20 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.07, 1.04 (2d, $J = 8.0$ Hz 6H, $\text{CHCH}(\text{CH}_3)_2$), 0.98, 0.94 (2d, $J = 7.0$ Hz 6H, $\text{CHCH}(\text{CH}_3)_2$).

Synthesis of α -naphthyl-(isopropoxy-L-methionine) phosphorochloridate (3.3x).

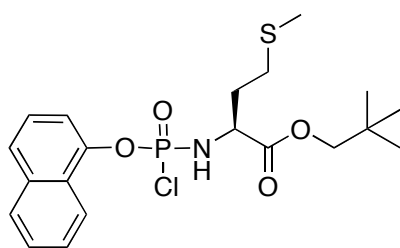


Prepared according to the Standard Procedure 3, from: L-methionine isopropyl ester hydrochloride salt (1.50 g, 6.59 mmol), α -naphthyl phosphorodichloridate (1.72g, 6.59 mmol) and Et_3N (1.19 ml, 13.17 mmol) in 50 ml of dry DCM. Product obtained as a white solid (1.84g, 67%).

^{31}P NMR (202 MHz, CDCl_3) δ 8.88, 8.80.

^1H NMR (500 MHz, CDCl_3) δ 8.15–8.12 (m, 1H, $\text{H}_8\text{-Naph}$), 7.89 (d, $J = 7.0$ Hz, 1H, $\text{H}_5\text{-Naph}$), 7.75 (d, $J = 7.0$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.65- 7.54 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.45 (t, $J = 7.5$ Hz, 1H, $\text{H}_3\text{-Naph}$), 5.16- 5.07 (m, 1H, $\text{OCH}(\text{CH}_3)_2$), 4.73- 4.68 (m, 1H, NH), 4.38- 4.31 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.66- 2.54 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.23- 2.10 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.08, 2.06 (2s, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.32, 1.31 (2d, $J = 7.0$ Hz, 3H, $\text{OCH}(\text{CH}_3)_2$), 1.29, 1.28 (2d, $J = 8.5$ Hz, 3H, $\text{OCH}(\text{CH}_3)_2$).

Synthesis of α -naphthyl-(2,2-dimethylpropoxy-L-methioninyl) phosphorochloridate (3.3y).



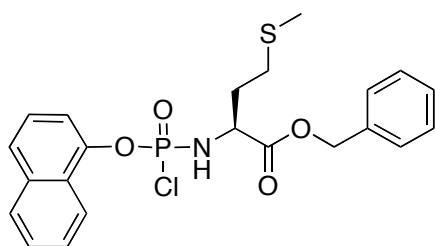
Prepared according to the Standard Procedure 3, from: L-methionine neopentyl ester tosylate salt (2.00 g, 5.11 mmol), α -naphthyl phosphorodichloridate (1.30 g, 5.11 mmol) and Et_3N (1.42 ml, 10.22 mmol) in 15 ml of dry DCM.

Product obtained as a white solid (0.92g, 41%).

^{31}P NMR (202 MHz, CDCl_3) δ 8.92, 8.74.

^1H NMR (500 MHz, CDCl_3) δ 8.15–8.11 (m, 1H, $\text{H}_8\text{-Naph}$), 7.87 (d, $J = 8.2$ Hz, 1H, $\text{H}_5\text{-Naph}$), 7.73 (d, $J = 8.85$, 1H, $\text{H}_4\text{-Naph}$), 7.67–.52 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.43 (t, $J = 8.0$, 1H, $\text{H}_3\text{-Naph}$), 4.97 (bs, 1H, NH), 4.48–4.41 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 3.93, 3.90, 3.89, 3.86 (2AB, $J_{\text{AB}} = 10.5$ Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$) 2.65–2.58 (m, 2H,), 2.25– 2.07 (m, 5H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$ and $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 0.99, 0.96 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of α -naphthyl-(2,2-dimethylpropoxy-L-methioninyl) phosphorochloridate (3.3z).



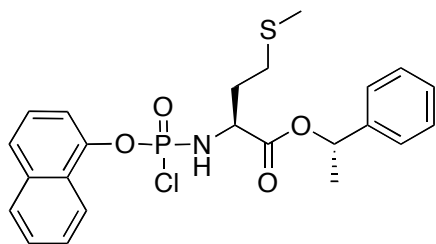
Prepared according to the standard procedure 3, from: L-methionine benzyl ester tosylate salt (2.00 g, 4.70 mmol), α -naphthyl phosphorodichloridate (1.23 g, 4.70 mmol) and Et_3N (1.31 ml, 9.40 mmol) in 30 ml of dry DCM.

Compound was obtained as clear, yellow, thick oil (1.67g, 74%).

^{31}P NMR (202 MHz, CDCl_3) δ 8.72, 8.63.

^1H NMR (500 MHz, CDCl_3) δ 8.13-8.08 (m, 1H, $\text{H}_8\text{-Naph}$), 7.90-7.88 (m, 1H, $\text{H}_5\text{-Naph}$), 7.75 (d, $J = 9.00$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.64–7.54 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.44 (t, $J = 8.20$ Hz, 1H, $\text{H}_3\text{-Naph}$), 7.35-7.31 (m, 5H, OCH_2Ph), 5.25, 5.22 (AB, $J_{\text{AB}} = 8.50$ Hz, 2H, OCH_2Ph), 4.75-4.69 (m, 1H, NH), 4.48 – 4.41 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.62 – 2.52 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.18– 1.96 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.03, 2.01 (2s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$).

Synthesis of α -naphthyl-(*S*-phenylethoxy-L-methioninyl) phosphorochloridate (3.3za).

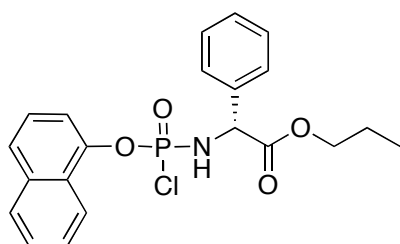


Prepared according to the standard procedure 3, from: L-methionine *S*-phenylethyl ester the tosylate salt (2.00 g, 4.70 mmol), α -naphthyl phosphorodichloridate (1.23 g, 4.70 mmol) and Et₃N (1.31 ml, 9.40 mmol) in 30 ml of dry DCM. Compound was obtained as clear, thick oil (1.67g, 74%).

³¹P NMR (202 MHz, CDCl₃) δ 8.64, 8.59

¹H NMR (500 MHz, CDCl₃) δ 8.12, 8.10 (2d, J = 8.00 Hz, 1H, H₈-Naph), 7.91-7.88 (m, 1H, H₅-Naph), 7.76 (d, J = 8.50 Hz, 1H, H₄-Naph), 7.65 – 7.55 (m, 3H, H₇, H₆, H₂-Naph), 7.46, 7.45 (2t, J = 8.00 Hz, 1H, H₃-Naph), 7.40-7.31 (m, 5H, OCH(CH₃)Ph), 6.00, 5.97 (2q, J = 7.50 Hz, 1H, OCH(CH₃)Ph), 4.58-4.47 (m, 1H, NH), 4.47 – 4.40 (m, 1H, CHCH₂CH₂SCH₃), 2.55–2.34 (m, 2H, CHCH₂CH₂SCH₃), 2.18– 1.96 (m, 5H, CHCH₂CH₂SCH₃ and CHCH₂CH₂SCH₃), 1.64, 1.60 (2d, J = 7.50 Hz, 3H, OCH(CH₃)Ph).

Synthesis of α -naphthyl-(propoxy-D-phenylglycynyl) phosphorochloridate (3.3zb).



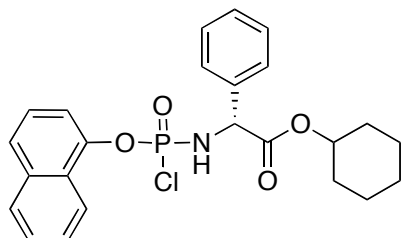
Prepared according to the standard procedure 3, from: D-phenylglycine propyl ester hydrochloride salt (2.00g, 8.71 mmol), α -naphthyl phosphorodichloridate (2.27, 8.71 mmol) and Et₃N (2.42 ml, 17.41 mmol) in 20 ml of dry DCM. Compound was obtained as clear, yellow, thick (2.15g, 59%).

³¹P NMR (202 MHz, CDCl₃) δ 7.72, 7.52.

¹H NMR (500 MHz, CDCl₃) δ 8.06 – 8.04 (m, 1H, H₈-Naph), 7.87, 7.81 (2d, J = 6.00 Hz, 1H, H₅-Naph), 7.81, 7.67 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.57–7.44 (m, 4H, H₇, H₆, H₂, H₃-Naph), 7.40–7.37 (m, 5H, CHPh), 5.37–5.24 (m, 1H, NH), 5.18–4.99 (m,

1H, *CHPh*), 4.15 (q, $J = 7.00$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.65-1.59 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 0.84 (t, $J = 7.00$ Hz, 3H, $\text{OCH}_2\text{CH}_2\text{CH}_3$).

Synthesis of α -naphthyl-(cyclohexoxy-D-phenylglycynyl) phosphorochloridate (3.3zc).



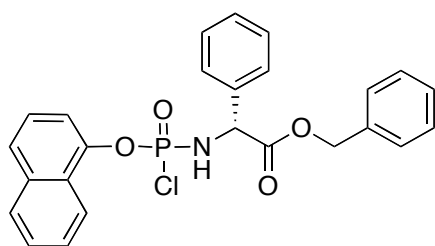
Prepared according to the standard procedure 3, from: D-phenylglycine cyclohexyl ester hydrochloride salt (2.00g, 7.41 mmol), α -naphthyl phosphorodichloridate (1.94g, 7.41 mmol) and Et_3N (2.06 ml, 14.83 mmol) in 15 ml of dry DCM.

Compound was obtained as yellow, thick oil yield (2.75g, 60%).

^{31}P NMR (202 MHz, CDCl_3) δ 7.82, 7.64.

^1H NMR (500 MHz, CDCl_3) δ 8.05, 7.88 (2d, $J = 8.00$ Hz, 1H, $\text{H}_8\text{-Naph}$), 7.87 (d, $J = 8.50$ Hz, 1H, $\text{H}_5\text{-Naph}$), 7.72, 7.66 (2d, $J = 8.0$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.58- 7.45 (m, 4H, H_7 , H_6 , H_2 , $\text{H}_3\text{-Naph}$), 7.40–7.37 (m, 5H, *CHPh*), 5.38–5.31 (m, 1H, NH), 5.25–5.17 (m, 1H, *CHPh*), 4.89–4.85 (m, 1H, OCH ester), 1.88–1.67 (m, 2H, CH_2 ester), 1.56-1.48 (m, 2H, CH_2 ester), 1.46-1.33 (m, 6H, 3 x CH_2 ester).

Synthesis of α -naphthyl-(benzoxo-D-phenylglycynyl) phosphorochloridate (3.3zd).



Prepared according to the standard procedure 3, from: D-phenylglycine benzyl ester tosylate salt (1.50g, 3.63 mmol), α -naphthyl phosphorodichloridate (0.95g, 3.63 mmol) and Et_3N (1.01 ml, 7.26 mmol) in 20 ml of dry DCM.

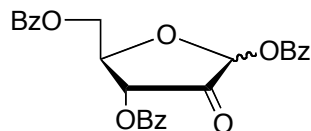
Compound was obtained as clear, yellow, thick oil (1.21g, 72%).

^{31}P NMR (202 MHz, CDCl_3) δ 7.60, 7.42.

^1H NMR (500 MHz, CDCl_3) δ 8.11–8.01 (m, 1H, $\text{H}_8\text{-Naph}$), 7.87, 7.81 (2d, $J = 8.5$ Hz, 1H, $\text{H}_5\text{-Naph}$), 7.72, 7.67 (2d, $J = 8.0$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.58–7.48 (m, 3H, H_7 , H_6 , H_2), 7.40–7.19 (m, 11H, $\text{H}_3\text{-Naph}$, *CHPh* and OCH_2Ph), 5.42–5.09 (m, 2H, NH and *CHPh*).

12.4 Experimental section - Chapter Four.

Synthesis of 1,3,5-tri-*O*-benzoyl-D-2-ketoribofuranose (4.2).

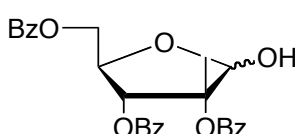
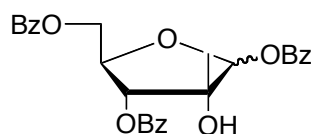


To a solution of Dess-Martine periodinane (7.13 g, 16.8 mmol) in anhydrous DCM (50 ml), was added 1,3,5-tri-*O*-benzoyl-D-ribofuranose (5.0 g, 11.2 mmol) at 0 °C. The mixture was allowed to warm up to ambient temperature and stirred for 16 h. The solvent was reduced under reduced pressure and the residu was triturated with diethyl ether (100 ml). The solid was filtered through MgSO₄ pad. The solvent was stirred with an equal volume of the Na₂S₂O₃ (12.5 g) solution of a saturated NaHCO₃. The otganic layer was separated, washed with brine and dried over anhydrous MgSO₄. The solvents was evaporated in vacuo and product was isolated as white foam. (4.57 g, 89%).

¹H NMR (500 MHz, CDCl₃) δ 8.35-8.15 (m, 6H, Bz), 7.56-7.44 (m, 3H, Bz), 7.41-7.31 (m, 6H, Bz), 6.15 (s, 1H, H₁), 5.80 (d, *J*= 8.8 Hz, 1H, H₃), 4.98-4.87 (m, 1H, H₄), 4.82-4.66 (m, 2H, H₅).

¹³C NMR (126 MHz, CDCl₃) δ 201.31 (C2), 166.02 (C=O, Bz), 165.57 (C=O, Bz), 165.20 (C=O, Bz), 134, 11, 133.95, 133.37, 130.56, 130.37, 129.80, 128.67, 128.55, 128.32 (C and CH, Bz), 91.45 (C1), 78.18 (C3), 72.05 (C4), 64.20 (C5).

Synthesis of 1,3,5-Tri-*O*-benzoyl-2-*C*-methyl-D-ribofuranose (4.3).

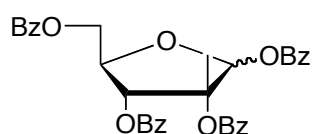


TiCl₄ (4.2 ml, 38.72 mmol) was added dropwise to anhydrous diethyl ether (120 ml) at -78 °C, followed by the addition of 3M solution of MeMgBr in diethyl ether (12.90 ml, 38.72 mmol). The reaction mixture was allowed to warm up to -10 °C and a solution of 1,3,5-tri-*O*-benzoyl-D-2-ketoribofuranose (**4.2**, 4.50 g, 9.68 mmol) in diethyl ether (13 ml). After 3 h, the reaction was quenched with 60 ml of water. The water layer was separted and extracted with diethyl ether (3x 50 ml). The combined organic layers were dried over anhydrous MgSO₄, and evaporated to dryness. Crude

mixture was purified on silica gel, using hexane/ethyl acetate 5:2 then 5:3 as eluent. The collected product consisted of a mixture of two species **4.3** and **4.4** (4.0g, 75%).

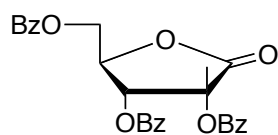
Synthesis of 2,3,5-Tri-*O*-benzoyl-2-*C*-methyl- D-ribofuranose (4.4**)** is reported after the synthesis of **4.7**, using **4.7** as a starting material.

Synthesis of 1,2,3,5-Tetra-*O*-benzoyl-2-*C*-methyl-D-ribofuranose (4.5**).**



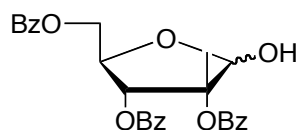
To a cold solution (5 °C) of **4.3** and **4.4** (11.3 g, 23.6 mmol), 4-dimethylaminopyridine (290 mg, 2.36 mmol) and triethylamine (16.4 ml, 117.9 mmol) in anhydrous tetrahydrofuran (120ml), benzoyl chloride (5.5 ml, 47.1 mmol) was added dropwise. The reaction mixture was stirred under nitrogen, at room temperature overnight. The reaction was quenched with ice-cold water (200 ml) and saturated aqueous solution of sodium bicarbonate. Tetrahydrofuran was removed under vacuum and the mixture was extracted with ethyl acetate (3x 50 ml). The organic layers were combined, washed with water and brine. Mixture was dried over anhydrous sodium sulfate. Solvent was removed under reduced pressure to give thick oil. This crude product was stirred with 200 ml of tert-butyl methyl ether for 5 minutes. Heptane (200 ml) and water (4.1 ml) were added and stirring was continued for two hours at room temperature. Solids were collected by vacuum filtration and filter cake was washed with mixture of heptane/tert-butyl methyl ether (1:1, 200 ml) and tert-butyl methyl ether (80 ml). Solid was dried in vacuum, to gave 6.75 g (63%) of desired product, as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 8.18 – 8.10 (m, 4H, Bz), 8.09–8.05 (m, 2H, Bz), 7.95 – 7.89 (m, 2H, Bz), 7.65-7.62 (m, 3H, Bz), 7.54–7.45 (m, 7H, Bz), 7.18-7.10 (m, 2H, Bz), 7.09, 7.08 (2s, 1H, H), 5.97-5.94 (m, 1H, H₃), 4.85–4.78 (m, 1H, H₄), 4.72-4.59 (m, 2H, H₅), 2.05, 1.97 (2s, 3H, CH₃).

Synthesis of 2,3,5-tri-*O*-benzoyl-2-*C*-methyl-D-ribonolactone (4.7).

A mixture of 2-*C*-methyl-D-ribo-1,4-lactone **4.6** (6.0 g, 37 mmol), 4-dimethylaminopyridine (0.90 g, 7.44 mmol) and triethylamine (69.5 ml, 500 mmol.) in 1,2-dimethoxyethane (100 ml) was stirred at 25 °C under argon atmosphere for 30 minutes. Reaction mixture was cooled down to 5 °C, and benzoyl chloride (19.32 ml, 166.46 mmol) was added dropwise. The mixture was stirred at room temperature for 2 hours. Ice-cold water (100 ml) was added to quench the reaction. Stirring was continued for 30 minutes. The formed white solids were collected by vacuum filtration and washed with cold water. Crude product was stirred with tert-butyl methyl ether (120 ml) at room temperature for 30 minutes, and then filtered, filter cake washed with tert-butyl methyl ether (2x 20 ml) and dried in vacuum to give 13.0 g (74% yield) of compound as a white solid. Compound was used for further synthesis without any purification.

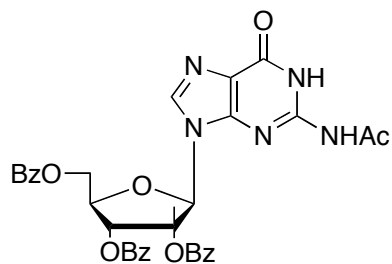
¹H NMR (500 MHz, CDCl₃) δ 8.07-8.05 (m, 2H, Bz), 7.96-7.94 (m, 2H, Bz), 7.76-7.74 (m, 2H, Bz), 7.61-7.57 (m, 1H, Bz), 7.45 (m, 4H, Bz), 7.36-7.33 (m, 2H, Bz), 7.21-7.18 (m, 2H, Bz), 5.51 (d, 1Hn H₃), 5.21-5.18 (m, 1H, H₄), 4.82-4.66 (m, 2H, H₅), 1.95 (s, 3H, 2'CCH₃).

Synthesis of 2,3,5-Tri-*O*-benzoyl-2-*C*-methyl- D-ribofuranose (4.4).

Red-A1 (65wt.% in toluene, 19.2 ml, 63 mmol.) in anhydrous toluene (19 ml) was stirred at 0 °C under argon atmosphere. A mixture of anhydrous ethanol (3.65 ml, 59.86 mmol) in anhydrous toluene (15.4 ml) was added dropwise to the Red-A1 solution. The resulting mixture was stirred at 0 °C for 15 minutes. Prepared that way reagent was added to a pre-cooled (-5 °C) solution of 2,3,5-tri-*O*-benzoyl-2-*C*-methyl-D-ribonolactone **3.7** (13.6 g, 28.6 mmol) in anhydrous toluene (96 ml) over a period of 15 minutes. The reaction mixture was stirred at -5 °C for 40 minutes. After that time, reaction was quenched by addition of acetone (1.9 ml), water (140 ml) and 1 N HCl (140 ml) at 0 °C. After that reaction mixture was allowed to warm to room temperature. 1 N HCl (~100 ml) was added to reach pH 2-3. The mixture was extracted with ethyl acetate (3x 200 ml). Organic layers were combined, washed with brine (350 ml) and dried over anhydrous

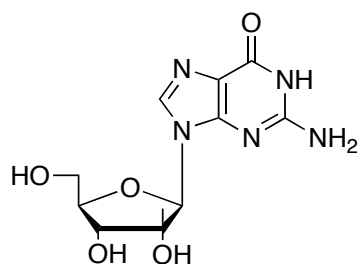
sodium sulphate. Solvent was removed under reduced give desired product in quantitative yield (~14 g). Product was used for subsequent step without further purifications.

Synthesis of *N*2-Acetyl-2',3',5'-tri-*O*-benzoyl-2'-*C*- β -methylguanosine (4.9).



A suspension of *N*2-acetylguanine **4.8** (2.00 g, 10.35 mmol) in dry pyridine (10 ml), and 1,1,1,3,3,3-hexamethyldisilazane (35 ml), was heated at reflux for 5 to 8 hours, to obtain a clear solution. Solvents were removed and dried carefully under high vacuum and maintained under argon. To the flask containing persilylated *N*2-acetylguanosine (10.35 mmol) was added *p*-xylene (100 ml) and 1,2,3,5-tetra-*O*-benzoyl-2-*C*- β -methylribofuranose **4.5** (2 g, 3.44 mmol). To the resulting mixture was added trimethylsilyl triflate (1.88 ml, 10.35 mmol) slowly with vigorous stirring at room temperature. After the reaction mixture was stirred under an argon atmosphere at 140-150 °C (reflux) for 6 h. The reaction mixture was cooled to room temperature and quenched with saturated aqueous sodium bicarbonate. The organic layer was separated, and the aqueous layer was extracted with dichloromethane (3x 150 ml). The organic layers were combined and dried over a magnesium sulfate. The magnesium sulfate was filtered off, and the solvent was removed under vacuum. The pure compound was obtained as a white precipitate from the mixture of chloroform and methanol (70% yield).

¹H NMR (500 MHz, CDCl₃) 12.04 (s, 1H, NH), 11.21 (s, 1H, NHAc), 8.17-7.23 (m, 16H, Bz), 6.69 (s, 1H, H_{1'}), 5.97 (d, 1H, *J* = 8.5 Hz, H_{3'}), 4.93-4.81 (m, 3H, H_{4'} and H_{5'}), 2.45 (s, 3H, acetyl), 1.50 (s, 3H, 2'*C*CH₃).

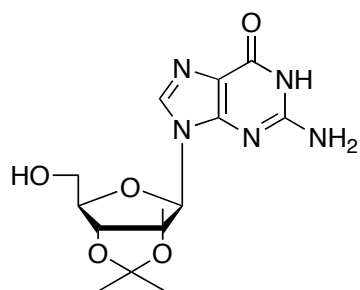
Synthesis of 2'-C- β -methylguanosine (4.10).

N-2-Acetyl-2',3',5'-tri-*O*-benzoyl-2'-C- β -methylguanosine **3.9** (1.00 g, 1.53 mmol) in methanol (20 ml) was saturated with ammonia for 30 min at -5 to -10 °C, then sealed and stirred at room temperature for 16 to 20 h. The solvent was removed by evaporation under vacuum. The residue was then dissolved in a small volume of hot methanol and precipitated by addition of chloroform. The solid was filtered off, washed with chloroform and dried to yield the deprotected nucleoside (90-100%).

^1H NMR (500 MHz, DMSO) δ 10.52 (bs, 1H, NH), 8.38 (s, 1H, H₈), 6.52 (bs, 2H, NH₂), 5.73 (s, 1H, H_{1'}), 5.24 (d, 1H, J = 6.7 Hz, 3'OH), 5.16 (t, 1H, J = 4.9 Hz, 5'OH), 5.03 (s, 1H, 2'OH), 4.01-3.96 (m, 1H, H_{3'}), 3.85-3.79 (m, 2H, H_{4'} and H_{5'a}), 3.79-3.72 (m, 1H, H_{5'b}), 0.81 (s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, DMSO) δ 156.70 (C6), 153.67 (C2), 150.74 (C4), 135.11 (C8), 116.32 (C5), 90.10 (C1'), 82.33 (C3'), 78.50 (C2'), 71.63 (C4'), 59.44 (C5'), 20.02 (2'CCH₃).

HRMS calculated for C₁₁H₁₅N₅O₅Na: 320.0986; found 320.0972.

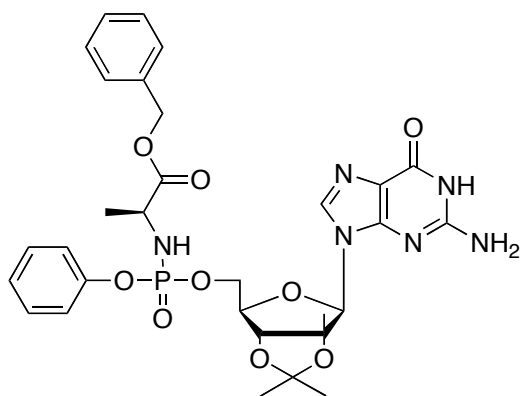
Synthesis of 2',3'-*O,O*-isopropylidene-2'-C- β -methylguanosine (4.11).

To a solution of 2'-C- β -methylguanosine **4.10** (1.50 g, 5.04 mmol) in anhydrous acetone (90 ml), at room temperature, perchloric acid (0.85 ml) was added dropwise and stirred overnight. Then, a saturated solution of NH₄OH was added dropwise to reach neutral pH. The solvent was removed under reduced pressure and the resulting solid was purified by column chromatography (CHCl₃/MeOH, 8:2), to yield the protected nucleoside **4.11** (1.61 g, 93%).

^1H NMR (500 MHz, DMSO) δ 10.61 (br s, 1H, NH), 7.92 (s, 1H, H₈), 6.48 (bs, 2H, NH₂), 6.01 (s, 1H, H_{1'}), 5.21 (t, 1H, J = 5.0 Hz, 5'OH), 4.58 (d, J = 2.7 Hz, 1H, H_{3'}), 4.18-4.16 (m, 1H, H_{4'}), 3.74-3.64 (m, 1H, H_{5'}), 1.54 (s, 3H, CH₃ isopropylidene), 1.38

(s, 3H, CH₃ isopropylidene), 1.16 (s, 3H, 2'CCH₃).

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine 5'-*O*-[α-phenyl-(benzoxy-L-alaninyl)] phosphate (4.12a).



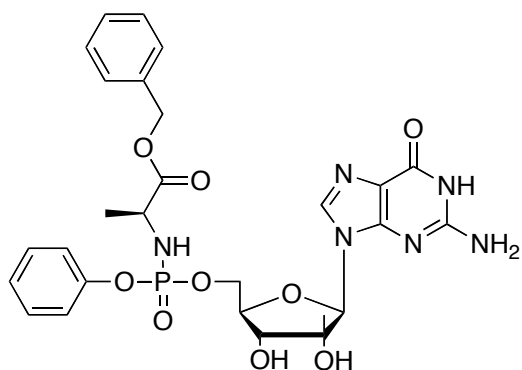
Prepared according to the Standard Procedure 4 from: 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine (**4.11**, 200 mg, 0.59 mmol), phenyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3a**, 419 mg, 1.18 mmol) *t*BuMgCl (1.18 ml, 1.18 mmol) in 3 ml of dry THF. The crude mixture was purified by column

chromatography, using CHCl₃/MeOH (2 to 8% gradient) as an eluent, to give a pure product **4.12a** as a white foam (115 mg, 30%).

³¹P NMR (202 MHz, MeOD) δ 4.63, 4.37.

¹H NMR (500 MHz, MeOD) δ 7.87, 7.85 (2s, 1H, H₈), 7.36-7.34 (m, 5H, OCH₂Ph and/or Ph), 7.32-7.28 (m, 5H, OCH₂Ph and/or Ph), 6.05, 6.03 (2s, 1H, H_{1'}), 5.17-5.12 (m, 2H, OCH₂Ph), 4.55-4.52 (m, 2H, H_{3'} and H_{5'a}), 4.47-4.38 (m, 2H, H_{5'b} and H_{4'}), 4.10-4.09 (m, 1H, CHCH₃), 1.61, 1.53, 1.39, 1.37 (4s, 3H, CH₃ isopropylidene), 1.33, 1.27 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.03, 1.00 (2s, 3H, 2'CCH₃).

Synthesis of 2'-*C*-β-methylguanosine 5'-*O*-[α-phenyl-(benzoxy-L-alaninyl)] phosphate (4.13a).



Prepared according to the Standard Procedure 5 from: 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine 5'-*O*-[α-naphthyl-(methoxy-L-alaninyl)] phosphate (**4.12a**, 115 mg, 0.177 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified

by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (9:1) as an eluent, to give a pure product **4.13a** as a white solid (68 mg, 62%).

^{31}P NMR (202 MHz, MeOD) δ 4.09, 3.93.

^1H NMR (500 MHz, MeOD) δ 7.99, 7.95 (2s, 1H, H_8), 7.32-7.23 (m, 10H, OCH_2Ph and Ph), 5.95, 5.93 (2s, 1H, $\text{H}_{1'}$), 5.14-5.09 (m, 2H, OCH_2Ph), 4.55-4.42 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{5'a}$), 4.23-4.17 (m, 2H, $\text{H}_{5'b}$ and $\text{H}_{4'}$), 4.07-4.01 (m, 1H, CHCH_3), 1.35, 1.33 (2d, $J=7.0$ Hz, 3H, CHCH_3), 1.00, 0.97 (2s, 3H, $2'\text{CCH}_3$).

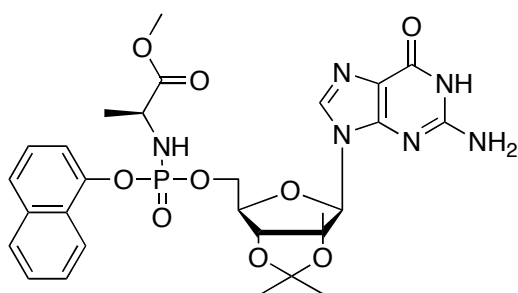
^{13}C NMR (126 MHz, MeOD) δ 174.83, 174.64 (2d, $^3J_{\text{C-C-N-P}} = 4.6$ Hz, C=O), 159.50, 159.49 (C_6), 155.34, 155.29 (C_2), 152.65, 152.64 (C_4), 152.16, 152.10 (ipso Ph), 137.48, 137.41 (C_8), 137.19, 137.16 (ipso OCH_2Ph), 131.16, 130.95, 130.82, 130.78, 129.90, 129.65, 129.59, 129.57, 129.31, 129.27, 129.18, 126.17, 121.52, 121.51 (Ph and OCH_2Ph), 118.05, 117.94 (C_5), 93.10, 92.78 ($\text{C}_{1'}$), 82.13, 81.96 (2d, $^3J_{\text{C-C-O-P}} = 8.3$ Hz, C_4'), 80.08, 80.01 (C_2'), 74.60, 74.11 (C_3'), 68.02, 67.98 (OCH_2Ph), 67.52, 66.61 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C_5'), 51.76, 51.62 (CHCH_3), 21.02 ($2'\text{CCH}_3$), 20.66, 20.43 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz CHCH_3).

HPLC (System 1) $t_R = 17.05, 17.25$ min.

MS (ES+) m/z : 637.18 ($\text{M}+\text{Na}^+$, 100%).

HRMS calculated for $\text{C}_{27}\text{H}_{31}\text{N}_6\text{O}_9\text{PNa}$: 637.1788; found. 637.1813.

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(methoxy-L-alaninyl)] phosphate (4.12b).



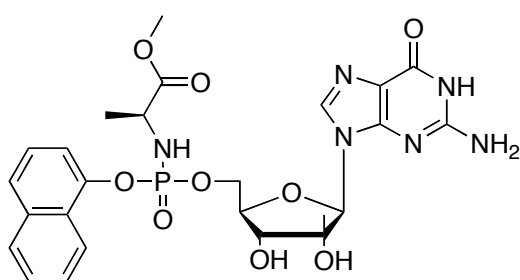
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C- β -methylguanosine (**4.11**, 300 mg, 0.889 mmol), α -naphthyl-(methoxy-L-alaninyl) phosphorochloridate

(**3.3a**, 582 mg, 1.78 mmol in 1M solution in THF) $t\text{BuMgCl}$ (1.78 ml, 1.78 mmol) in 3 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (2 to 8% gradient) as an eluent, to give a pure product **4.12b** as a white foam (17.8 mg, 32%).

^{31}P NMR (202 MHz, MeOD) δ 4.49, 4.40.

^1H NMR (500 MHz, MeOD) δ 8.17 (t, $J = 8.0$ Hz, 1H, H₈-Naph), 7.87-7.65 (m, 2H, H₅-Naph and H₈), 7.71-7.69 (m, 1H, H₄-Naph), 7.58-7.48 (m, 3H, H₇, H₆, H₂-Naph), 7.43-7.41 (m, 1H, H₃-Naph), 6.08, 6.07 (2s, 1H, H_{1'}), 4.54-4.50 (m, 2H, H_{3'} and H_{5'a}), 4.48-4.36 (m, 2H, H_{5'b} and H_{4'}), 4.10-4.09 (m, 1H, CHCH₃), 3.61 (s, 3H, OCH₃), 1.59, 1.55, 1.39, 1.36 (4s, 3H, CH₃ isopropylidene), 1.03, 1.00 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(methoxy-L-alaninyl)] phosphate (4.13b).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O'-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(methoxy-L-alaninyl)] phosphate (**4.12b**, 178 mg, 0.283 mmol) in 10 ml of 60% CH₃COOH in water at 90

°C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13b** as a white solid (93 mg, 55%).

^{31}P NMR (202 MHz, MeOD) δ 4.38, 4.25.

^1H NMR (500 MHz, MeOD) δ 8.18 (m, 1H, H₈-Naph), 7.93-7.83 (m, 2H, H₅-Naph and H₈), 7.69-7.64 (m, 1H, H₄-Naph), 7.56-7.49 (m, 3H, H₇, H₆, H₂-Naph), 7.42-7.38 (m, 1H, H₃-Naph), 5.94, 5.93 (2s, 1H, H_{1'}), 4.57-4.51 (m, 2H, H_{5'}), 4.29-4.23 (m, 2H, H_{3'} and H_{4'}), 4.10-3.98 (m, 1H, CHCH₃), 3.56, 3.54 (2s, 3H, OCH₃), 1.31-1.28 (m, 3H, CHCH₃), 1.01, 0.99 (2s, 3H, 2'CCH₃).

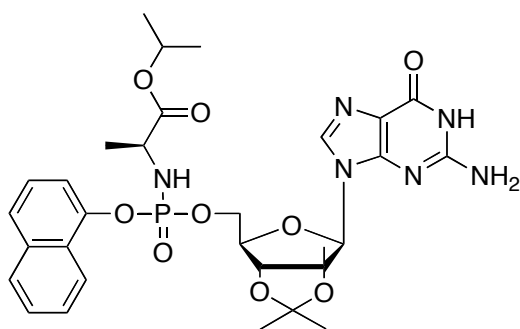
^{13}C NMR (126 MHz, MeOD) δ 174.62 (d, $^3J_{\text{C-C-N-P}} = 4.6$ Hz, C=O), 174.41 (d, $^3J_{\text{C-C-N-P}} = 5.6$ Hz, C=O), 159.57, 159.55 (C6), 155.30, 155.27 (C2), 152.67, 152.66 (C4), 148.01, 147.97 (2d, $^2J_{\text{C-O-P}} = 2.8$ Hz, ipso Naph), 137.99, 137.79 (C8), 136.27, 136.24 (C10-Naph), 128.85, 128.80 (CH-Naph), 127.90, 127.85 (C9-Naph), 127.75, 127.47, 126.51, 126.49, 125.97, 125.94, 122.80, 122.75 (CH-Naph), 118.05 (C5), 116.22, 116.16 (2d, $^3J_{\text{C-C-O-P}} = 3.3$ Hz, C2-Naph), 93.25, 93.15 (C1'), 82.24 (d, $^3J_{\text{C-C-O-P}} = 7.9$, C4'), 82.15 (d, $^3J_{\text{C-C-O-P}} = 8.7$, C4'), 80.02, 79.97 (C2'), 74.79, 74.59 (C3'), 67.94,

67.59 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 61.97, 61.92 (OCH₃), 51.87, 51.79 (CHCH₃), 21.96, 21.89 (2'CCH₃), 20.73, 20.47 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃).

HPLC (System 1) $t_R = 14.67, 15.04$ min.

HRMS calculated for C₂₅H₂₉N₆O₉PNa: 611.1627; found. 611.1632.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(isopropoxy-L-alaninyl)] phosphate (4.12c).



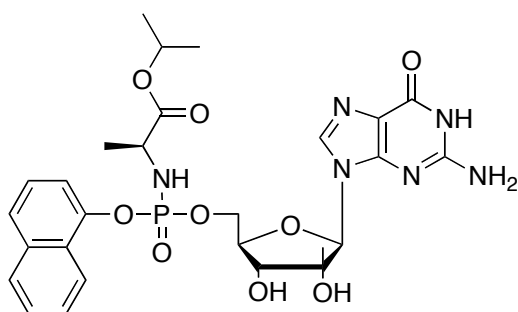
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 200 mg, 0.593 mmol), α-naphthyl (isopropoxy-L-alaninyl) phosphorochloridate (**3.3b**, 422mg, 1.19 mmol) tBuMgCl (1.19 ml, 1.19 mmol) in

3 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as an eluent, to give a pure product **4.12c** as a white foam (202 mg, 52%).

^{31}P NMR (202 MHz, CDCl₃) δ 4.49, 4.46.

^1H NMR (500 MHz, MeOD) δ 8.21, 8.17 (2d, $J = 8.0$ Hz, 1H, H₈-naph) 7.95-7.79 (m, 2H, H₅-Naph and H₈), 7.75-7.70 (m, 1H, H₄-Naph), 7.58-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.48-7.41 (m, 1H, H₃-Naph), 6.09, 6.08 (2s, 1H, H_{1'}), 4.99-4.92 (m, 1H, OCH(CH₃)₂), 4.63-4.52 (m, 2H, H_{3'}, H_{5'a}), 4.46-4.40 (m, 2H, H_{5'b}, H_{4'}), 4.05-4.01 (m, 1H, CHCH₃), 1.61, 1.59 (2s, 3H, CH₃ isopropylidene), 1.42, 1.40 (2d, $J = 6.0$ Hz, 3H, CHCH₃), 1.37, 1.35 (2s, 3H, CH₃ isopropylidene), 1.25, 1.23 (2d, $J = 2.0$ Hz, 3H, OCH(CH₃)₂), 1.21, 1.20 (2d, $J = 2.0$ Hz, 3H, OCH(CH₃)₂), 1.04, 1.00 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(isopropoxy-L-alaninyl)] phosphate (4.13c).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O'-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(isopropoxy-L-alaninyl)] phosphate (**4.12c**, 202 mg, 0.308 mmol) in 10 ml of 60% CH₃COOH

in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13c** as a white solid (100 mg, 53%).

³¹P NMR (202 MHz, MeOD) δ 4.39, 4.26.

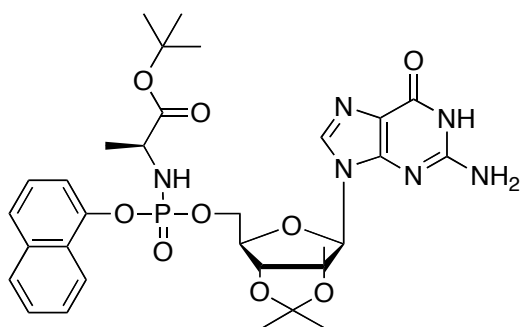
¹H NMR (500 MHz, MeOD) δ 8.20-8.17 (m, 1H, H₈-Naph), 7.89-7.84 (m, 2H, H₈, H₅-Naph), 7.69, 7.67 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.54- 7.48 (m, 3H, H₇, H₆, H₂-Naph), 7.41, 7.38 (2t, J = 8.0 Hz, 1H, H₃-Naph), 5.95, 5.94 (2s, 1H, H_{1'}), 5.10-4.99 (m, 1H, OCH(CH₃)₂), 4.67-4.57 (m, 2H, H_{5'}), 4.31-4.21 (m, 2H, H_{3'}, H_{4'}), 4.04-4.94 (m, 1H, CHCH₃) 1.28 (m, 3H, CHCH₃), 1.15-1.11 (m, 6H, OCH(CH₃)₂), 1.01 and 0.98 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.61 (d, $^3J_{C-C-N-P}$ = 4.6 Hz, C=O), 174.38 (d, $^3J_{C-C-N-P}$ = 5.6 Hz, C=O), 159.52, 159.49 (C6), 155.29, 155.26 (C2), 152.65, 152.64 (C4), 148.01, 147.96 (ipso Naph), 137.95, 137.73 (C8), 136.27, 136.24 (C10-Naph) 128.85, 128.80 (CH-Naph), 127.90, 127.85 (2d, $^3J_{C-C-O-P}$ = 1.5 Hz, C9-Naph), 127.75 127.47 126.51, 126.47 125.97, 125.93, 122.82, 122.77 (CH-Naph), 118.06 (C5), 116.22, 116.15 (2d, $^3J_{C-C-O-P}$ = 3.2 Hz, C2-Naph), 93.25, 93.17 (C1'), 82.23 (d, $^3J_{C-C-O-P}$ = 7.9 Hz, C4'), 82.14 (d, $^3J_{C-C-O-P}$ = 8.7 Hz, C4'), 80.03, 79.98 (C2'), 74.79, 74.59 (C3'), 70.20, 70.18 (OCH(CH₃)₂), 67.94 (d, $^2J_{C-O-P}$ = 5.2 Hz, C5'), 67.58 (d, $^2J_{C-O-P}$ = 4.6 Hz, C5'), 51.87, 51.79 (CHCH₃), 30.76 (OCH(CH₃)₂), 21.96, 21.89 (2'CCH₃), 20.73, 20.58 (2d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃).

HPLC (System 1) t_R = 14.71, 15.07 min.

HRMS calculated for C₂₇H₃₃N₆O₉PNa: 639.1944; found 639.1934.

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(*tert*-butoxy-L-alaninyl)] phosphate (4.12d).

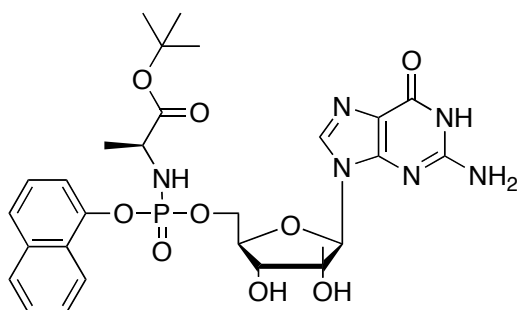


Prepared according to the Standard Procedure 4 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine (**4.11**, 200 mg, 0.593 mmol), α -naphthyl (*tert*-butoxy-L-alaninyl) phosphorochloridate (**3.3d**, 436 mg, 1.19 mmol) tBuMgCl (1.19 ml, 1.19 mmol) in 3 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as eluent, to give a pure product **4.12d** as a white foam (170 mg, 43%).

³¹P NMR (202 MHz, CDCl₃) δ 4.63, 4.52.

¹H NMR (500 MHz, MeOD) δ 8.22-8.17 (m, 1H, H₈-naph) 7.94-7.87 (m, 2H, H₅-Naph and H₈), 7.76-7.70 (m, 1H, H₄-Naph), 7.59-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.48-7.41 (m, 1H, H₃-Naph), 6.09, 6.07 (2s, 1H, H_{1'}), 4.62, 4.58 (2d, *J* = 3.5 Hz, 1H, H_{3'}), 4.55-4.52 (m, 1H, H_{5'a}), 4.47-4.40 (m, 2H, H_{5'b}, H_{4'}), 4.05-4.01 (m, 1H, CHCH₃), 1.61, 1.58 (2s, 3H, CH₃ isopropylidene), 1.43, 1.41 (2s, 9H, OC(CH₃)₃), 1.35, 1.34 (2d, *J* = 6.0 Hz, 3H, CHCH₃), 1.37, 1.35 (2s, 3H, CH₃ isopropylidene), 1.02, 0.99 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(*tert*-butoxy-L-alaninyl)] phosphate (4.13d).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(*tert*-butoxy-L-alaninyl)] phosphate (**4.12d**, 170 mg, 0.253 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13c** as a white solid (97 mg, 60%).

^{31}P NMR (202 MHz, CDCl_3) δ 4.51, 4.28.

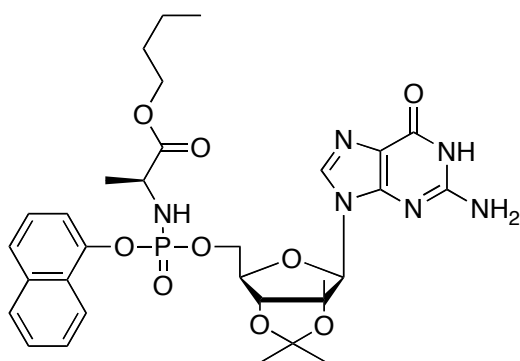
^1H NMR (500 MHz, MeOD) δ 8.21-8.18 (m, 1H, $\text{H}_8\text{-naph}$) 7.88-7.84 (m, 2H, $\text{H}_5\text{-Naph}$ and H_8), 7.71-7.66 (m, 1H, $\text{H}_4\text{-Naph}$), 7.53-7.47 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.42-7.37 (m, 1H, $\text{H}_3\text{-Naph}$), 5.93 (s, 1H, $\text{H}_{1'}$), 4.63-4.57 (m, 2H, H_5'), 4.31-4.22 (m, 2H, H_3' and H_4'), 3.94-3.88 (m, 1H, CHCH_3), 1.37, 1.34 (2s, 9H, $\text{OC}(\text{CH}_3)_3$), 1.30, 1.29 (2d, $J = 7.5$ Hz, 3H, CHCH_3), 1.01, 0.98 (2s, 3H, $2'\text{CCH}_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.60, 174.35 (C=O), 159.49, 159.41 (C_6), 155.29, 155.26 (C_2), 152.66, 152.65 (C_4), 148.00, 147.95 (ipso Naph), 137.95, 137.77 (C_8), 136.27, 136.24 ($\text{C}_{10}\text{-Naph}$) 128.85, 128.80 (CH-Naph), 127.93, 127.87 ($\text{C}_9\text{-Naph}$), 127.74, 127.45, 126.50, 126.44, 125.94, 125.88, 122.80, (CH-Naph), 118.10 (C_5), 116.21, 116.08 (2d, $^3J_{\text{C-C-O-P}} = 3.2$, $\text{C}_2\text{-Naph}$), 93.30, 93.20 ($\text{C}_{1'}$), 82.68, 82.64 ($\text{OC}(\text{CH}_3)_3$), 82.23, 82.12 (2d, $^3J_{\text{C-C-O-P}} = 7.9$ Hz, C_4'), 79.98, 79.95 (C_2'), 74.79, 74.58 (C_3'), 67.90, 67.54 (C_5'), 52.30 (CHCH_3), 28.19, 28.14 ($\text{OC}(\text{CH}_3)_3$), 20.88, 2.84 ($2'\text{CCH}_3$), 20.62, 20.57 (CHCH_3).

HPLC (System 1) $t_R = 15.63$, 15.87 min.

HRMS calculated for $\text{C}_{28}\text{H}_{35}\text{N}_6\text{O}_9\text{P}$: 630.2278; found 630.2215.

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(butoxy-L-alaninyl)] phosphate (4.12e).



Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C- β -methylguanosine (**4.11**, 250 mg, 0.741 mmol), α -naphthyl-(butoxy-L-alaninyl) phosphorochloridate (**3.3d**, 0.548mg, 1.48 mmol), $t\text{BuMgCl}$ (1.48 ml, 1.48 mmol) in 3 ml of dry THF.

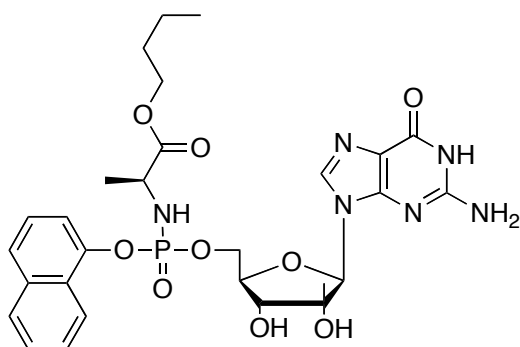
Crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (2 to 8%, gradient) as en eluent, to give a pure product **4.12e** as white foam (200 mg, 42%).

^{31}P NMR (202 MHz, MeOD) δ 4.48, 4.46.

^1H NMR (500 MHz, MeOD) δ 8.21, 8.16 (2d, $J = 9.00$ MHz, 1H, $\text{H}_8\text{-Naph}$), 7.92-7.88 (m, 1H, $\text{H}_5\text{-Naph}$), 7.80-7.71 (m, 2H, $\text{H}_4\text{-Naph}$ and H_8), 7.60- .52 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.48-7.41 (m, 1H, $\text{H}_3\text{-Naph}$), 6.10, 6.08 (2s, 1H, $\text{H}_{1'}$), 4.64- 4.52 (m, 2H, H_3' ,

H_{5'a}), 4.47-4.39 (m, 2H, H_{5'b}, H_{4'}), 4.10-4.01 (m, 3H, CHCH₃ and OCH₂CH₂CH₂CH₃), 1.61, 1.59 (2s, 3H, CH₃ isopropylidene), 1.57–1.53 (m, 2H m, 2H, OCH₂CH₂CH₂CH₃), 1.40-1.32 (m, 8H, CHCH₃, CH₃ isopropylidene, and OCH₂CH₂CH₂CH₃), 1.04, 1.01 (2s, 3H, 2'CCH₃), 0.89, 0.87 (2t, *J* = 8.5 Hz, 3H, OCH₂CH₂CH₂CH₃).

Synthesis of 2'-C-β-methylguanosine 5'-O-[α-naphthyl-(butoxy-L-alaninyl)] phosphat (4.13e).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(butoxy-L-alaninyl)] phosphate (**4.12e**, 200 mg, 0.298 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. Crude mixture was purified

by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13e** as a white solid (62 mg, 30%).

³¹P NMR (202 MHz, MeOD) δ 4.37, 4.20.

¹H NMR (500 MHz, MeOD) δ 8.20-8.16 (m, 1H, H₈-Naph), 7.90–7.88 (2s, 1H, H₈), 7.87-7.83(m, 1H, H₅-Naph), 7.70-7.65 (m, 1H, H₄-Naph), 7.53–7.47 (m, 3H, H₇, H₆, H₂-Naph), 7.41-7.36 (m, 1H, H₃-Naph), 5.95, 5.94 (2s, 1H, H_{1'}), 4.67-4.57 (m, 2H, H_{5'}), 4.30- 4.22 (m, 2H, H_{3'}, H_{4'}), 4.07- 4.00 (m, 1H, CHCH₃) 3.98- 3.96 (m, 2H, OCH₂CH₂CH₂CH₃), 1.50–1.43 (m, 2H m, 2H, OCH₂CH₂CH₂CH₃), 1.32-1.27 (m, 3H: CHCH₃, OCH₂CH₂CH₂CH₃), 1.00, 0.98 (2s, 3H, 2'CCH₃), 0.87-0.83 (m, 3H, OCH₂CH₂CH₂CH₃).

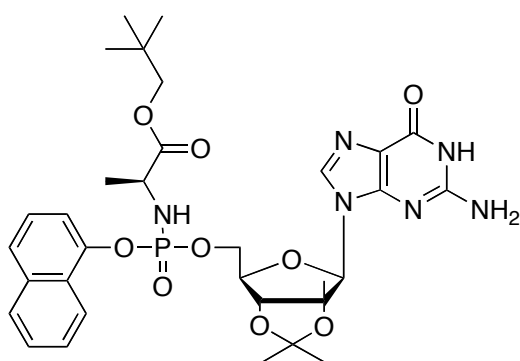
¹³C NMR (126 MHz, MeOD) δ 175.10 (d, ³*J*_{C-C-N-P} = 4.5 Hz, C=O), 174.87 (d, ³*J*_{C-C-N-P} = 5.6 Hz, C=O ester), 159.49, 159.47 (C6), 155.30, 155.26 (C2), 152.64 (C4), 148.01, 147.96 (2d, *J* = 2.9 Hz, ipso Naph), 137.90, 137.71 (C8), 136.28, 136.24 (C10-Naph), 128.85, 128.79, 127.91, 127.85 (C9-Naph), 127.47, 126.51, 126.46, 125.95, 125.91, 122.81, 122.76 (CH-Naph), 118.05 (C5), 116.21, 116.10 (2d, ³*J*_{C-C-O-P} = 3.2 Hz, C2-Naph), 93.24, 93.16 (C1'), 82.21, 82.10 (2d, ³*J*_{C-C-O-P} = 8.6 Hz, C4'), 80.02, 79.98 (C2'), 74.76, 74.56 (C3'), 67.92, 67.53 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 66.17,

66.16 (OCH₂CH₂CH₂CH₃), 51.76, 51.72 (CHCH₃), 31.65, 31.62 (OCH₂CH₂CH₂CH₃), 20.71, 20.47 (2d, ³J_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.38 (2'CCH₃), 20.03 (OCH₂CH₂CH₂CH₃), 14.02, 14.00 (OCH₂CH₂CH₂CH₃)

HPLC (System 1) t_R=16.27, 16.88 min.

HRMS calculated for C₂₈H₃₅N₆O₉PNa: 653.2101; found 653.2095.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (4.12f).



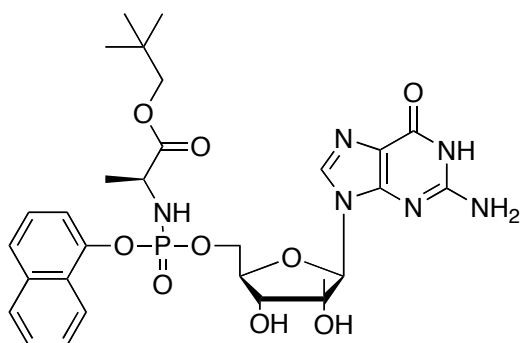
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 1.50 g, 4.45 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 3.40 g, 8.89 mmol), *t*BuMgCl (8.89 ml, 8.89 mmol, 1M solution in THF) in 15 ml of dry THF.

The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as an eluent, to give a pure product **4.12f** as white foam (1.73g, 57%).

³¹P NMR (202 MHz, MeOD) δ 4.51, 4.47.

¹H NMR (500 MHz, MeOD) δ 8.21-8.15 (m, 1H, H₈-Naph), 7.91- 7.67 (m, 1H, H₅-Naph), 7.79- 7.70 (m, 2H, H₄-Naph and H₈), 7.58-7.51 (m, 3H, H₇, H₆, H₂-Naph), 7.47- 7.41 (m, 1H, H₃-Naph), 6.09, 6.07 (2s, 1H, H_{1'}), 4.67- 4.53 (m, 2H, H_{3'}, H_{5'a}), 4.45- 4.39 (m, 2H, H_{5'b}, H_{4'}), 4.14- 4.10 (m, 1H, CHCH₃), 3.83, 3.81, 3.77, 3.75 (2AB, J_{AB}= 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.61, 1.58 (2s, 3H, CH₃ isopropylidene), 1.41-1.39 (m, 6H, CH₃ isopropylidene, CHCH₃), 1.03, 1.00 (2s, 3H, 2'CCH₃), 0.89, 0.88 (2s, 9H, OCH₂C(CH₃)₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (4.13f).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -Naphthyl-(2,2dimethylpropoxy-L-alaninyl)] phosphate (**4.12f**, 1.73 g, 2.53 mmol) in 100 ml of 60% CH₃COOH in water at 90 °C overnight. Crude mixture

was purified by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13f** as a white solid (100mg, 53%).

³¹P NMR (202 MHz, MeOD) δ 4.33, 4.25.

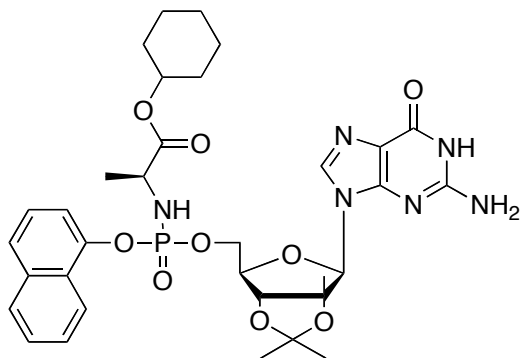
¹H NMR (500 MHz, MeOD) δ 8.20- 8.17 (m, 1H, H₈-Naph), 7.89- 7.85 (m, 2H, H₅ naph and H₈), 7.71- 7.67 (m, 1H, H₄-Naph), 7.54 – 7.47 (m, 3H, H₇, H₆, H₂-Naph), 7.43- 7.37 (m, 1H, H₃-Naph), 5.93 (s, 1H, H_{1'}), 4.67- 4.53 (m, 2H, H_{5'}), 4.28- 4.20 (m, 2H, H_{3'} and H_{4'}), 4.11- 4.03 (m, 1H, CHCH₃), 3.76, 3.74, 3.65, 3.62 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.36- 1.33 (m, 3H, CHCH₃), 1.00, 0.98 (2s, 3H, 2'CCH₃), 0.88, 0.87 (s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.05 (d, $^3J_{C-C-N-P}$ = 4.6, C=O), 174.80 (d, $^3J_{C-C-N-P}$ = 5.6, C=O), 159.52, 159.49 (C6), 155.39, 155.35 (C2), 152.65 (C4), 148.01, 147.95 (d, $^2J_{C-O-P}$ = 3.3, ipso Naph), 137.91, 137.72 (C8), 136.30, 136.26 (C10-Naph), 128.85, 128.79 (CH-Naph), 127.90, 127.85 (C9-Naph), 127.75, 127.74, 127.48, 127.47, 126.51, 126.45, 125.97, 125.93, 122.81, 122.76 (CH-Naph), 118.05 (C5), 116.23, 116.16 (2d, $^3J_{C-C-O-P}$ = 3.2, C2-Naph), 93.26, 93.18 (C1'), 82.23, 82.12 (2d, $^3J_{C-C-O-P}$ = 7.5, C4'), 80.00, 79.94 (C2'), 75.38, 75.36 (OCH₂C(CH₃)₃), 74.81, 74.60 (C3'), 68.05, 67.63 (2d, $^2J_{C-O-P}$ = 5.0, C5'), 51.80, 51.73 (CHCH₃), 32.26, 32.23 (OCH₂C(CH₃)₃), 31.31 (CHCH₃), 26.73, 26.70 (OCH₂C(CH₃)₃), 20.86, 20.81 (2'CCH₃).

HPLC (System 1) t_R = 16.87, 17.13 min.

HRMS calculated for C₂₉H₃₇N₆O₉PNa: 667.2257; found 667.2249.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (4.12g).



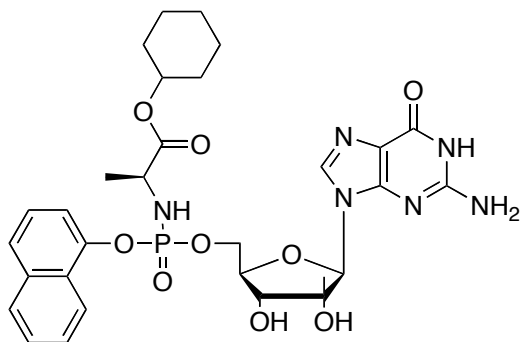
Prepared according to the Standard Procedure 4 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine (**4.11**, 474 mg, 1.40 mmol), α -naphthyl-(cyclohexoxy-L-alaninyl) phosphorochloridate (**3.3h**, 1.11 g, 2.81 mmol), *t*BuMgCl (2.81 ml, 2.81 mmol) in

6 ml of dry THF. Crude mixture was purified by column chromatography CHCl₃/MeOH (2 to 8%, gradient) to give a pure product **4.12g** as white foam (980 mg, 100%).

³¹P NMR (202 MHz, MeOD) δ 4.49, 4.48.

¹H NMR (500 MHz, MeOD) δ 8.20-8.18 (m, 1H, H₈-Naph), 7.92-7.86 (m, 2H, H₅-Naph and H₈), 7.75-7.68 (m, 1H, H₄-Naph), 7.58-7.51 (m, 3H, H₇, H₆, H₂-Naph), 7.48- 7.43 (m, 1H, H₃-Naph), 6.09, 6.07 (2s, 1H, H_{1'}), 4.73-4.69 (m, 2H, H_{3'}, H_{5'a}), 4.60- 4.39 (m, 3H, H_{5'b}, H_{4'}, OCH ester), 4.07-4.03 (m, 1H, CHCH₃), 1.77-1.68 (m, 4H, 2x CH₂ ester), 1.39-1.36 (m, 15H, 2x CH₃ isopropylidene, CHCH₃, 3x CH₂ ester), 1.04, 1.00 (2s, 3H, 2'CCH₃).

Synthesis of 2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (4.13g).



Prepared according to the Standard Procedure 5 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (**4.12g**, 980 mg, 1.40 mmol) in 30 ml of 60% CH₃COOH in water at 90 °C overnight. The crude

mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as eluent, to give a pure product **4.13g** as a white solid (285 mg, 31%).

³¹P NMR (202 MHz, MeOD) δ 4.39, 4.22.

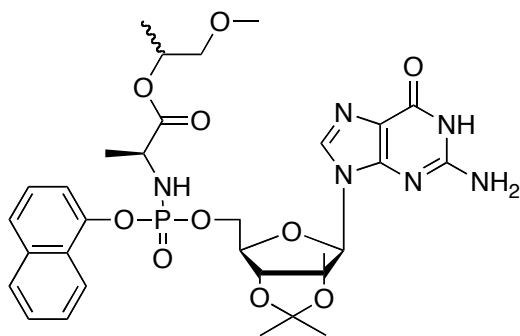
¹H NMR (500 MHz, MeOD) δ 8.19-8.16 (m, 1H, H₈-Naph), 7.90-7.80 (m, 2H, H₅-Naph and H₈), 7.67-7.63 (m, 1H, H₄-Naph), 7.53-7.46 (m, 3H, H₇, H₆, H₂-Naph), 7.40-7.34 (m, 1H, H₃-Naph), 5.95 (s, 1H, H_{1'}), 4.68- 4.59 (m, 3H, H_{5'}, OCH ester), 4.31-4.24 (m, 2H, H_{3'}, H_{4'}), 4.05-3.97 (m, 1H, CHCH₃), 1.71-1.61 (m, 4H, CH₂ ester), 1.35-1.20 (m, 9H, CHCH₃, 3x CH₂ ester), 1.00, 0.98 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.27, 174.22 (C=O), 159.40 (C6), 155.29 (C2), 152.64, 152.61 (C4), 148.04, 147.98 (ipso Naph), 137.86, 137.68 (C8), 136.31, 136.27 (C10-Naph), 128.84, 128.78 (CH-Naph), 127.92, 127.85 (C9-Naph), 127.74, 127.46, 126.51, 126.44, 125.95, 125.88, 122.82, 122.76 (CH-Naph), 118.09 (C5), 116.20, 116.10 (2d, ³J_{C-C-O-P} = 3.2 Hz, C2-Naph), 93.28, 93.18 (C1'), 82.23, 82.10 (2d, ³J_{C-C-O-P} = 8.5 Hz, C4'), 79.98, 79.95 (C2'), 79.47 (OCH ester), 74.79, 74.58 (C3'), 67.99, 67.56 (2d, ²J_{C-O-P} = 5.0 Hz, C5'), 51.87, 51.80 (CHCH₃), 32.37, 32.29, 26.36, 24.57 (CH₂ ester), 20.74 (d, ³J_{C-C-N-P} = 6.2 Hz, CHCH₃), 20.51 (d, ³J_{C-C-N-P} = 7.5, CHCH₃), 20.35, 20.32 (2'CCH₃).

HPLC (System 1) t_R = 17.36, 17.54 min.

HRMS calculated for C₃₀H₃₇N₆O₉PNa: 679.2257; found 679.2267.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(1-methoxy-2-propoxy-L-alaninyl)] phosphate (4.12h).



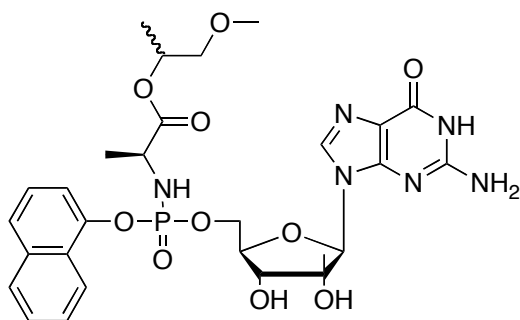
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 250 mg, 0.741 mmol), α-naphthyl-(1-methoxy-2-propoxy-L-alaninyl) phosphorochloridate (572 mg, 1.48

mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in 4 ml of dry THF. Crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as eluent, to give a pure product **4.12h** as white foam (273 mg, 54%).

^{31}P NMR (202 MHz, MeOD) δ 4.52, 4.45, 4.44.

^1H NMR (500 MHz, MeOD) δ 8.20-8.17 (m, J = 7.50 Hz, 1H, H₈-Naph), 7.90, 7.89, 7.87, 7.86 (4s, 1H, H₈), 7.78-7.70 (m, 2H, H₅-Naph, H₄-Naph), 7.59-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.46-7.43 (m, 1H, H₃-Naph), 6.09, 6.08, 6.07 (3s, 1H, H_{1'}), 5.08-5.02 (m, 1H, OCH(CH₃)CH₂OCH₃), 4.63-4.51 (m, 2H, H_{3'} and H_{5'a}), 4.47-4.38 (m, 2H, H_{5'b} and H_{4'}), 4.11-4.05 (m, 1H, CHCH₃), 3.41-3.37 (m, 2H, OCH(CH₃)CH₂OCH₃), 3.32, 3.31, 3.30, 3.29 (4s, 3H, OCH(CH₃)CH₂OCH₃), 1.61, 1.58 (2 s, 3H, isopropylidene) 1.39, 1.37 (2d, J = 7.5 Hz, 3H, CHCH₃), 1.37, 1.36 (2 s, 3H, isopropylidene), 1.19-1.14 (m, 3H, OCH(CH₃)CH₂OCH₃), 1.03, 0.99 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(1-methoxy-2-propoxy-L-alaninyl)] phosphate (4.13h).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(1-methoxy-2-propoxy-L-alaninyl)] phosphate (**4.12h**, 273 mg, 0.398 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude

mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as eluent, to give a pure product **4.13h** as a white solid (70.1 mg, 27%).

^{31}P NMR (202 MHz, MeOD) δ 4.35, 4.32, 4.28.

^1H NMR (500 MHz, MeOD) δ 8.20-8.17 (m, 1H, H₈-Naph), 7.90, 7.87 (2s, 1H, H₈), 7.86-7.83 (m, 1H, H₅-Naph), 7.68-7.63 (m, 1H, H₄-Naph), 7.54-7.47 (m, 3H, H₇, H₆, H₂-Naph), 7.40-7.35 (m, 1H, H₃-Naph), 5.95, 5.94 (2s, 1H, H_{1'}), 5.00-4.92 (m, 1H, OCH(CH₃)CH₂OCH₃), 4.67-4.58 (m, 2H, H_{5'}), 4.31-4.23 (m, 2H, H_{3'} and H_{4'}), 4.06-3.99 (m, 1H, CHCH₃), 3.34-3.33 (m, 2H, OCH(CH₃)CH₂OCH₃), 3.27-3.26 (m, 3H, OCH(CH₃)CH₂OCH₃), 1.32, 1.31 (2d, J = 7.5 Hz, 3H, CHCH₃), 1.13-1.09 (m, 3H, OCH(CH₃)CH₂OCH₃), 1.00, 0.98 (2s, 3H, 2'CCH₃).

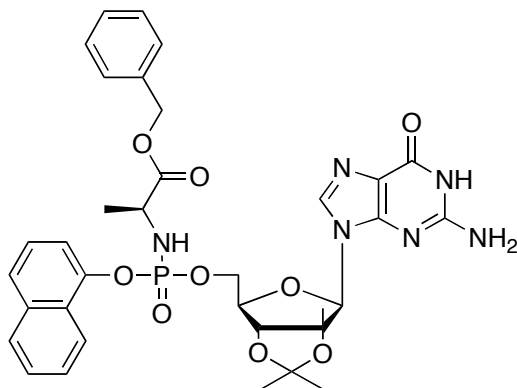
^{13}C NMR (126 MHz, MeOD) δ 174.42, 174.32 (2d, $^3J_{\text{C-C-N-P}}$ = 6.7 Hz, C=O), 159.49, 159.46 (C6), 155.28, 155.26 (C2), 152.64 (C4), 148.01, 147.96 (2d, $^2J_{\text{C-O-P}}$ = 2.8, ipso

Naph), 137.95, 137.72 (C8), 136.27, 136.24 (C10-Naph), 128.85, 128.80 (CH-Naph), 127.90, 127.85 (C9-Naph), 127.76, 127.49, 126.53, 126.48, 125.95, 122.86, 122.84, 122.77 (CH-Naph), 118.05 (C5), 116.22, 166.16 (2d, $^3J_{C-C-O-P} = 3.8$ Hz, C2-Naph), 93.24, 93.16 (C1'), 82.21, 82.12 (2d, $^3J_{C-C-O-P} = 8.8$ Hz, C4'), 80.01, 79.96 (C2'), 75.88, 75.74, 75.70 (OCH(CH₃)CH₂OCH₃), 74.80, 74.57 (C3'), 71.73, 71.72, 71.64, 71.59 (OCH(CH₃)CH₂OCH₃), 67.94, 67.53 (2d, $^2J_{C-O-P} = 3.8$ Hz, C5'), 59.38, 59.35, 59.33, 59.30 (OCH(CH₃)CH₂OCH₃), 51.80, 51.78 (CHCH₃), 20.71, 20.49 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃), 20.40, 20.38 (2'CCH₃), 16.65, 16.61, 16.58, 16.57 (OCH(CH₃)CH₂OCH₃).

HPLC (System 1) $t_R = 13.77, 14.23, 14.37$ min.

HRMS calculated for C₂₈H₃₅N₆O₁₀PNa: 669.2019; found 669.2027.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-L-alaninyl)] phosphate (4.12i).



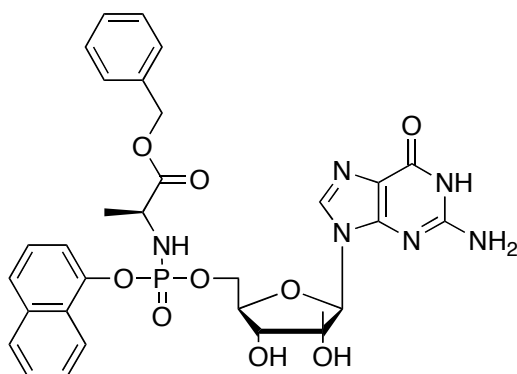
Prepared according to the Standard Procedure 4 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 200 mg, 0.592 mmol), α-naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (513 mg, 1.18 mmol), *t*BuMgCl (1.18 ml, 1.18 mmol) in 4 ml of dry THF. Crude

mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as an eluent, to give a pure product **4.12i** as white foam (210 mg, 50%).

^{31}P NMR (202 MHz, MeOD) δ 4.54, 4.38.

^1H NMR (500 MHz, MeOD) δ 8.22–8.12 (m, 1H, H₈-Naph), 7.94–7.85 (m, 2H, H₅-Naph and H₈), 7.79–.69 (m, 2H, H₅- and H₄-Naph), 7.59–7.48 (m, 3H, H₇, H₆, H₂-Naph), 7.43–7.41 (m, 1H, H₃-Naph), 7.33–7.29 (m, 5H, OCH₂Ph), 6.08, 6.06 (2s, 1H, H₁'), 5.16–5.08 (m, 2H, OCH₂Ph), 4.60, 4.52 (2d, $J = 3.3$ Hz, 1H, H₃'), 4.53–4.39 (m, 2H, H₅'), 4.36–4.31 (m, 1H, H₄'), 4.18–4.09 (m, 1H, CHCH₃), 1.60, 1.58 (2s, 3H, CH₃ isopropylidene), 1.42–1.38 (m, 6H, CHCH₃ and CH₃ isopropylidene), 1.02, 0.99 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(benzoxy-L-alaninyl)] phosphate (4.13i).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(1-methoxy-2-propoxy-L-alaninyl)] phosphate (**4.12i**, 210 mg, 0.298 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as eluent, to give a pure product **4.13i** as a white solid (78 mg, 39%).

³¹P NMR (202 MHz, MeOD) δ 4.27, 4.26.

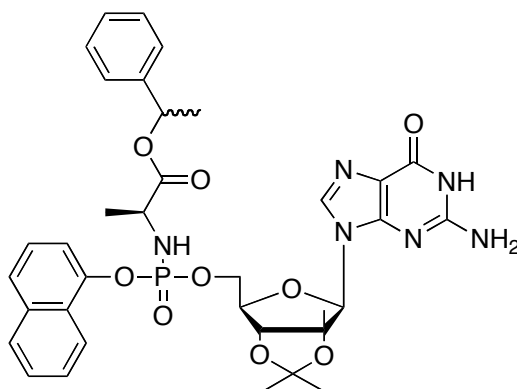
¹H NMR (500 MHz, MeOD) δ 8.19-8.17 (m, 1H, H₈-Naph), 7.87-7.80 (m, 2H, H₅-Naph and H₈), 7.67-7.64 (m, 1H, H₄-Naph), 7.53-7.42 (m, 3H, H₇, H₆, H₂-Naph), 7.37-7.35 (m, 1H, H₃-Naph), 7.30-7.21 (m, 5H, OCH₂Ph), 5.95, 9.94 (2s, 1H, H₁'), 5.07-4.93 (m, 2H, OCH₂Ph), 4.64-4.51 (m, 2H, H_{3'} and H_{5'a}), 4.32-4.19 (m, 2H, H_{4'} and H_{5'b}), 4.15-4.04 (m, 1H, CHCH₃), 1.32-1.28 (m, 3H, CHCH₃), 0.99, 0.97 (2s, 3H, 2'CCH₃).

HPLC (System 1) t_R = 16.11, 16.43 min.

MS (ES+) m/z : 687.19 (M+Na⁺, 100%).

HRMS calculated for C₃₁H₃₃N₆O₁₀PNa: 687.1953, found: 687.1922.

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(*R,S*-phenylethoxy-L-alaninyl)] phosphate (4.12j).



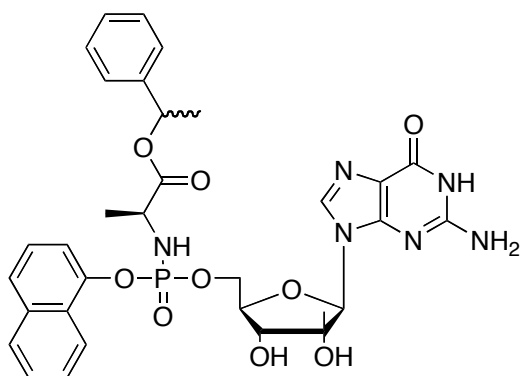
Prepared according to the Standard Procedure 4 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine (250mg, 0.741 mmol), α -naphthyl-(*R,S*-phenylethoxy-L-alaninyl) phosphorochloridate (620 mg, 1.48 mmol)

*t*BuMgCl (1.48 ml, 1.48 mmol) in 4 ml of dry THF. Crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as en eluent, to give a pure product **4.12j** as white foam (310 mg, 58%).

³¹P NMR (202 MHz, MeOD) δ 4.52, 4.45, 4.42.

¹H NMR (500 MHz, MeOD) δ 8.21-8.12 (m, 1H, H₈-Naph), 7.91-7.88 (m, 1H, H₅-Naph), 7.78-7.69 (m, 2H, H₄-Naph, H₈), 7.58-7.49 (m, 4H, H₇, H₆, H₃, H₂-Naph), 7.42-7.34 (m, 5H, OCH(CH₃)Ph), 6.08, 6.06, 6.05, 6.03 (4s, 1H, H₁'), 5.88-5.83 (m, 1H, OCH(CH₃)Ph), 4.58-4.47 (m, 2H, H₃', H_{5'}_a), 4.42-4.24 (m, 2H, H_{5'}_b, H₄'), 4.15-4.08 (m, 1H, CHCH₃), 1.52-1.48 (m, 3H, OCH(CH₃)Ph), 1.44-1.33 (m, 9, CHCH₃, 2x CH₃ isopropylidene), 1.01, 0.99, 0.98, 0.93 (4s, 3H, 2'CCH₃).

Synthesis of 2'-C-β-methylguanosine 5'-O-[α-Naphthyl-(R,S-phenylethoxy -L-alaninyl)] phosphate (4.13j).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(R,S-phenylethoxy-L-alaninyl)] phosphate (**4.12j**, 310 mg, 0.431 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column

chromatography, using CHCl₃/MeOH (9:1) as en eluent, to give a pure product **4.13j** as a white solid (78.6 mg, 27%).

³¹P NMR (202 MHz, MeOD) δ 4.37, 4.32, 4.29, 4.16.

¹H NMR (500 MHz, MeOD) δ 8.20-8.13 (m, 1H, H₈-Naph), 7.91-7.79 (m, 2H, H₅-Naph and H₈), 7.68-7.62 (m, 1H, H₄-Naph), 7.54-7.17 (m, 9H, H₇, H₆, H₃, H₂-Naph and OCH(CH₃)Ph), 5.96, 5.95, 5.93, 5.92 (4s, 1H, H₁'), 5.78-5.67 (m, 1H, OCH(CH₃)Ph), 4.65-4.53 (m, 2H, H₅'), 4.30-4.20 (m, 2H, H₃', H₄'), 4.12-4.04 (m, 1H, CHCH₃), 1.42-1.33 (m, 6H, OCH(CH₃)Ph and CHCH₃), 1.00, 0.98, 0.97, 0.95 (4s, 3H, 2'CCH₃).

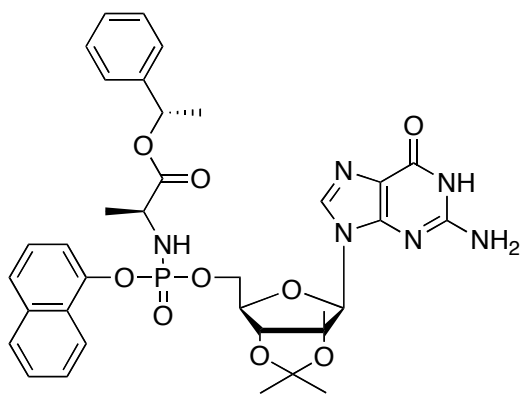
^{13}C NMR (126 MHz, MeOD) δ 174.29, 174.02 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, C=O), 159.54, 159.51 (C6), 155.28, 155.26 (C2), 152.66, 152.64 (C4), 147.96, 147.90 (2d, $^2J_{\text{C-O-P}} = 2.5$ Hz, ipso Naph), 142.88, 142.82, 142.63, 142.61 (ipso OCH(CH₃)Ph), 137.93, 137.88, 137.69 (C8), 136.26, 136.23, 136.20 (C10-Naph), 129.54, 129.52, 129.51, 128.96, 128.87, 128.84, 128.78 (OCH(CH₃)Ph and CH-Naph), 127.89, 127.85 (Naph-C9), 127.78, 127.76, 127.52, 127.50, 127.04, 126.99, 126.53, 126.46, 125.97, 122.84, 122.75 (OCH(CH₃)Ph and CH-Naph), 118.02 (C5), 116.29, 116.19 (2d, $^3J_{\text{C-C-O-P}} = 3.7$, C2-Naph), 93.22, 93.16, 93.10 (C1'), 82.24, 82.10 (2d, $^3J_{\text{C-C-N-P}} = 8.8$ Hz, C4'), 80.04, 80.03, 79.98 (C2'), 74.85, 74.79, 74.74, 74.69 (C3'), 74.50 (OCH(CH₃)Ph), 68.01, 67.94, (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 51.97, 51.94, 51.78, 51.73 (CHCH₃), 22.71, 22.61, 22.54, 22.47 (OCH(CH₃)Ph), 20.72, 20.59, 20.50, 20.45 (4d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH₃), 20.44 (2'CCH₃).

HPLC (System 1) t_R =17.11, 17.67, 17.77, 17.96 min.

HRMS calculated for C₃₂H₃₅N₆O₉PNa: 701.2101; found 701.2102.

CHN calculated for C₃₂H₃₅N₆O₉P x H₂O: C55.17, H5.35, N 12.06; found. C 55.24, H 5.27, N 11.57,

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(S-phenylethoxy -L-alaninyl)] phosphate (4.12k).



Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C- β -methylguanosine (**4.11**, 12 g, 35.57 mmol), 1.5 eq of α -naphthyl-(S-phenylethoxy-L-alaninyl) phosphorochloridate (**3.31**, 22.29 g, 53.36 mmol), *t*BuMgCl (71.14 ml, 71.14 mmol) in 150 ml of dry THF. The crude mixture

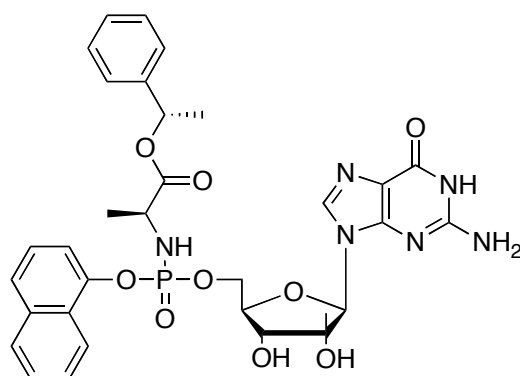
was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as en eluent, to give a pure product **4.12k** as white foam (20g, 78%).

^{31}P NMR (202 MHz, MeOD) δ 4.54, 4.45.

^1H NMR (500 MHz, MeOD) δ 8.21-8.12 (m, 1H, H₈-Naph), 7.91-7.88 (m, 1H, H₅-Naph), 7.78-7.69 (m, 2H, H₄-Naph, H₈), 7.58-7.49 (m, 4H, H₇, H₆, H₃, H₂-Naph),

7.42-7.34 (m, 5H, OCH(CH₃)Ph), 6.08, 6.06, (2s, 1H, H_{1'}), 5.88-5.83 (m, 1H, OCH(CH₃)Ph), 4.58-4.47(m, 2H, H_{3'}, H_{5a'}), 4.42- 4.24 (m, 2H, H_{5'b}, H_{4'}), 4.15-4.08 (m, 1H, CHCH₃), 1.52-1.48 (m, 3H, OCH(CH₃)Ph), 1.44-1.33 (m, 9, CHCH₃, 2x CH₃ isopropylidene), 0.99, 0.98, (2s, 3H, 2'CCH₃).

Synthesis of 2'-C-β-methylguanosine 5'-O-[α-naphthyl-(S-phenylethoxy-L-alaninyl)] phosphate (4.13k).



Prepared according to the Standard Procedure 5 from: 2',3'-O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(S-phenylethoxy-L-alaninyl)] phosphate (**4.12k**, 20 g, 27.84 mmol) in 500 ml of 60% CH₃COOH in water at 90 °C overnight. The crude

mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13k** as a white solid (4.05 g, 21%).

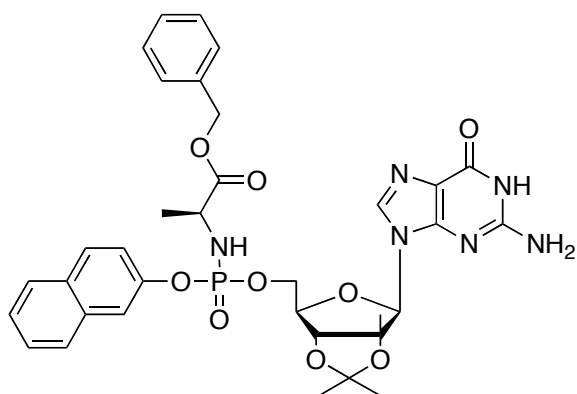
³¹P NMR (202 MHz, MeOD) δ 4.34, 4.29.

¹H NMR (500 MHz, MeOD) δ 8.20-8.13 (m, 1H, H₈-Naph), 7.91-7.79 (m, 2H, H₅-Naph and H₈), 7.68-7.62 (m, 1H, H₄-Naph), 7.54-7.17 (m, 9H, H₇, H₆, H₃, H₂-Naph and OCH(CH₃)Ph), 5.95, 5.93, (2s, 1H, H_{1'}), 5.78-5.67 (m, 1H, OCH(CH₃)Ph), 4.65-4.53 (m, 2H, H_{5'}), 4.30-4.20 (m, 2H, H_{3'}, H_{4'}), 4.12-4.04 (m, 1H, CHCH₃), 1.42-1.33 (m, 6H, OCH(CH₃)Ph and CHCH₃), 0.99, 0.98 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.24 (d, ³J_{C-C-N-P} = 4.5, C=O), 174.99 (d, ³J_{C-C-N-P} = 5.7, C=O), 159.50, 159.49 (C6), 155.30, 155.26 (C2), 152.68 (C4), 147.99, 147.93 (2d, ²J_{C-O-P} = 2.5 Hz, ipso Naph), 142.87, 142.82 (ipso OCH(CH₃)Ph), 137.95, 137.70 (C8), 136.27, 136.24, (C10-Naph) 129.53, 128.94, 128.92, 128.87, 128.82 (CH-Naph, OCH(CH₃)Ph), 127.89, 127.84 (C9-Naph), 127.77, 127.51, 127.04, 127.01, 126.52, 126.48, 125.99, 125.95, 122.83, 122.76 (CH-Naph, OCH(CH₃)Ph), 118.06 (C5), 116.27, 116.19 (2d, ³J_{C-C-O-P} = 3.1 Hz, C2-Naph), 93.25, 93.12 (C1'), 82.24, 82.10 (2d, ³J_{C-C-O-P} = 8.5 Hz, C4'), 80.02, 79.96 (C2'), 74.85, 74.79 (C3'), 74.54 (OCH(CH₃)Ph), 68.02, 67.52 (2d, ²J_{C-O-P} = 5.0 Hz, C5'), 51.81, 51.68

(CHCH₃), 22.65, 22.55 (OCH(CH₃)Ph), 20.54, 2.40 (2d, $^3J_{C-C-N-P}$ = 6.0 Hz, CHCH₃), 20.35, 2.31 (2'CCH₃).

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[β-naphthyl-(benzoxy-L-alaninyl)] phosphate (4.12l).



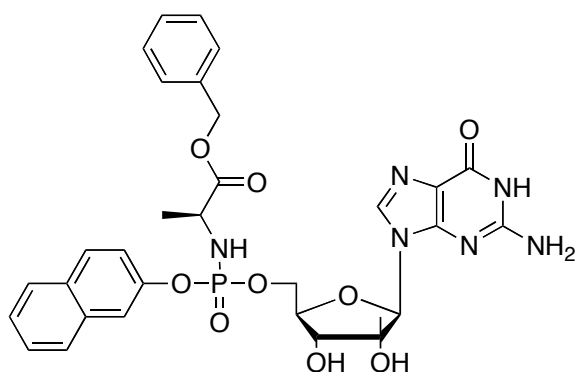
Prepared according to the Standard Procedure 4 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 250 mg, 0.741 mmol), β-naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (598 mg, 1.48 mmol) *t*BuMgCl (1.48 ml, 1.48 mmol) in 6 ml of dry THF. The crude

mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as en eluent, to give a pure product **4.12l** as white foam (212.5 mg, 41%).

^{31}P NMR (202 MHz, MeOD) δ 4.32, 4.22.

^1H NMR (500 MHz, MeOD) δ 7.89-7.81 (m, 4H, CH-Naph), 7.75 (s, 1H, H₈), 7.53-7.44 (m, 3H, CH-Naph), 7.32-7.29 (m, 5H, OCH₂Ph), 6.12, 6.10 (2s, 1H, H_{1'}), 5.14-5.08 (m, 2H, OCH₂Ph), 4.67, 4.60 (2d, J = 3.5 Hz, 1H, H_{3'}), 4.54-4.30 (m, 3H, H_{5'}, H_{4'}), 4.15-4.08 (m, 1H, CHCH₃), 1.60, 1.58 (2s, 3H, CH₃ isopropylidene), 1.39 – 1.36 (m, 6H, CH₃ isopropylidene and CHCH₃), 1.15, 1.05 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C-β-methylguanosine 5'-O-[β-naphthyl-(benzoxy-L-alaninyl)] phosphate (4.13l).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[β-naphthyl-(benzoxy-L-alaninyl)] phosphate (**4.12l**, 212.5 mg, 0.301 mmol) in 10

ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13l** as a white solid (53 mg, 26%).

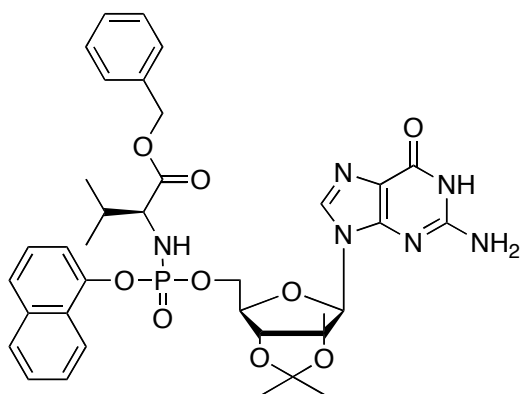
³¹P NMR (202 MHz, MeOD) δ 4.19, 4.16.

¹H NMR (500 MHz, MeOD) δ 7.88-7.81 (m, 3H, CH-Naph), 7.76-7.72 (m, 2H, H₈), 7.48-7.37 (m, 3H, CH-Naph), 7.29-7.23 (m, 5H, OCH₂Ph), 5.93 (s, 1H, H_{1'}), 5.10-5.00 (m, 2H, OCH₂Ph), 4.57-4.50 (m, 2H, H_{5'}), 4.24-4.05 (m, 3H, H_{3'}, H_{4'}, CHCH₃), 1.36, 1.33 (2d, *J* = 7.5 Hz, 3H, CHCH₃), 0.99, 0.91 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.78, 174.66 (C=O), 159.42 (C6), 155.31, (C2), 149.70, 149.65 (C4), 137.18, 137.14 (ipso OCH₂Ph), 135.28 (ipso Naph), 132.40 (C9-Naph), 130.91, 130.85 (C10-Naph), 130.60, 129.81, 129.68, 129.61, 129.54, 129.51, 129.25, 129.22, 129.22, 129.21, 129.13, 128.77, 128.53, 128.51, 127.80, 127.79, 126.58, 121.49, 121.45 (CH-Naph and OCH₂Ph), 117.90 (C5), 117.86, 117.82 (CH-Naph), 93.13, 93.05 (C1'), 82.10, 81.95 (2d, ³*J*_{C-C-O-P} = 8.5 Hz, C4'), 80.05, 79.98 (C2'), 74.55, 74.02 (C3'), 67.94, 67.88 (OCH₂Ph), 67.56 (d, ²*J*_{C-O-P} = 6.8 Hz, C5'), 67.68 (d, ²*J*_{C-O-P} = 4.7 Hz, C5'), 51.76, 51.67 (CHCH₃), 20.55, 20.35 (2d, ³*J*_{C-C-N-P} = 7.0 Hz, CHCH₃), 20.27, 20.23 (2'CCH₃).

HPLC (System 1) t_R = 15.89, 16.32 min.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-L-valinyl)] phosphate (4.12m).



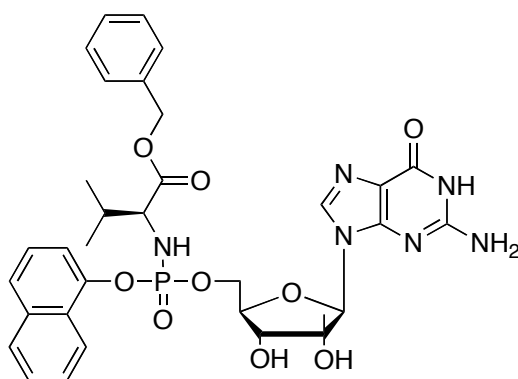
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 250 mg, 0.741 mmol), α-naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3t**, 640mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in 4 ml of dry THF. The crude mixture

was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as eluent, to give a pure product **4.12m** as white foam (225 mg, 41%).

^{31}P NMR (202 MHz, MeOD) δ 5.44, 5.30.

^1H NMR (500 MHz, MeOD) δ 8.20-8.17 (m, 1H, H₈-Naph), 7.92-7.87 (m, 1H, H₅-Naph), 7.76 (s, 1H, H₈), 7.74-7.70 (m, 1H, H₄-Naph), 7.56-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.45-7.39 (m, 1H, H₃-Naph), 7.34-7.28 (m, 5H, OCH₂Ph), 6.07, 6.06 (2s, 1H, H_{1'}), 5.15-4.99 (m, 2H, OCH₂Ph), 4.62-4.52 (m, 1H, H_{3'}, H_{5'a}), 4.44-4.31 (m, 2H, H_{5'b}, H_{4'}), 3.87-3.77 (m, 1H, CHCH(CH₃)₂), 2.12-2.01 (m, 1H, CHCH(CH₃)₂), 1.60, 1.58, 1.39, 1.38 (4s, 6H, 2x CH₃ isopropylidene), 1.03, 0.94 (2s, 3H, 2'CCH₃), 0.90 – 0.85 (m, 6H, CHCH(CH₃)₂).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(benzoxy-L-valinyl)] phosphate (4.13m).



Prepared according to the Standard Procedure 5 from: 2', 3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(benzoxy-L-valinyl)] phosphate (**4.12m**, 225 mg, 0.307 mmol) in 10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13m** as a white solid (47.8 mg, 24%).

^{31}P NMR (202 MHz, MeOD) δ 5.13, 4.98.

^1H NMR (500 MHz, MeOD) δ 8.18 (d, 1H, J = 8.0 MHz, H₈ naph), 7.88-7.84 (m, 2H, H₅-Naph and H₈), 7.71-7.67 (m, 1H, H₄-Naph), 7.55-7.49 (m, 3H, H₇, H₆, H₂-Naph), 7.39-7.35 (m, 1H, H₃-Naph), 7.28-7.26 (m, 5H, OCH₂Ph), 5.92, 5.91 (2s, 1H, H_{1'}), 5.05- 4.92 (m, 2H, OCH₂Ph), 4.58-4.54 (m, 2H, H_{5'}), 4.29-4.19 (m, 2H, H_{3'}, H_{4'}), 3.81-3.76 (m, 1H, CHCH(CH₃)₂), 2.05-1.94 (m, 1H, CHCH(CH₃)₂), 1.01, 0.96 (2s, 3H, 2'CCH₃), 0.85-0.79 (m, 6H, CHCH(CH₃)₂).

^{13}C NMR (126 MHz, MeOD) δ 174.05, 173.78 (2d, $^3J_{\text{C-C-N-P}}$ = 3.0 Hz, C=O), 159.50, 159.48 (C6), 155.28, 155.25 (C2), 152.63 (C4), 148.02, 147.97 (ipso Naph), 138.02, 137.80 (C8), 137.05, 137.02 (ipso OCH₂Ph), 136.27, 136.22 (C10-Naph), 129.51,

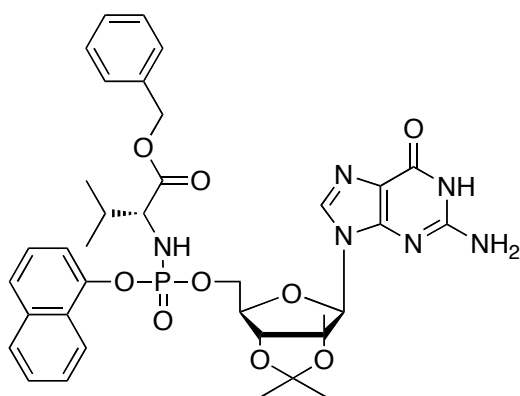
129.50, 129.49, 129.44, 129.30, 128.84, 128.78 (CH-Naph and OCH₂Ph), 127.90, 127.85 (2d, ³J_{C-C-O-P} = 2.5 Hz, Naph-C9), 127.76, 127.74, 127.48, 126.53, 126.52, 126.45, 126.44, 125.94, 122.87, 122.83 (CH-Naph and OCH₂Ph), 118.11 (C5), 116.25, 116.18 (2d, ³J_{C-C-O-P} = 3.0 Hz, C2-Naph), 93.35, 93.21 (C1'), 82.32, 82.16 (2d, ³J_{C-C-O-P} = 7.5 Hz, C4'), 79.97, 79.94 (C2'), 74.85, 74.66 (C3'), 68.30, 67.84 (2d, ²J_{C-O-P} = 5.0 Hz, C5'), 67.78, 67.73 (OCH₂Ph), 62.05, 62.02 (CHCH(CH₃)₂), 33.31, 33.19 (2d, ³J_{C-C-N-P} = 7.5 Hz, CHCH(CH₃)₂), 20.43, 19.48 (2'CCH₃), 18.39, 18.26 (CHCH(CH₃)₂).

HPLC (System 1) t_R = 18.85, 19.28 min.

HRMS calculated for C₃₃H₃₇N₆O₉PNa: 715.2257; found 715.2236.

CHN calculated for C₃₃H₃₇N₆O₉P + 1.0 H₂O: C 55.77, H 5.53, N 11.83; found: C 55.39, H 5.37, N, 11.83.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-D-valinyl)] phosphate (4.12n).



Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 250 mg, 0.741 mmol), α-naphthyl-(benzoxy-D-valinyl) phosphorochloridate (**3.2v**, 640 mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in 4 ml of dry THF.

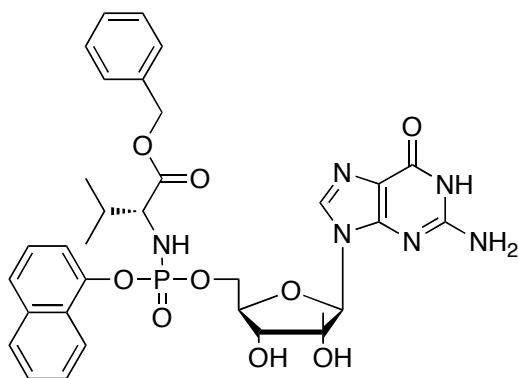
The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as an eluent, to give a pure product **4.12n** as white foam (331mg, 61%).

³¹P NMR (202 MHz, MeOD) δ 5.71, 5.09.

¹H NMR (500 MHz, MeOD) δ 8.23-8.21, 8.16-8.14 (2m, 1H, H₈ naph), 7.90-7.87 (m, 1H, H₅ naph), 7.75, 7.74 (2s, 1H, H₈), 7.72- 7.67 (m, 1H, H₄-Naph), 7.56-7.52 (m, 3H, H₇, H₆, H₂-Naph), 7.45, 7.36 (2d, *J* = 8.0 Hz, 1H, H₃-Naph), 7.30-7.26 (m, 5H, OCH₂Ph), 6.05, 6.04 (s, 1H, H_{1'}) 5.14-5.02 (m, 2H, OCH₂Ph), 4.59-4.33 (m, 4H, H_{3'}, H_{4'}, H_{5'}) 3.86-3.80 (m, 1H, CHCH(CH₃)₂), 2.09-2.01 (m, 1H, CHCH(CH₃)₂), 1.59,

1.57 (2s, 3H, CH₃ isopropylidene), 1.38, 1.36 (2s, 3H, CH₃ isopropylidene), 1.00, 0.96 (2s, 3H, 2'CCH₃), 0.90-0.85 (m, 6H, CHCH(CH₃)₂).

Synthesis of 2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-D-valinyl)] phosphate (4.13n).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-D-valinyl)] phosphate (**4.12n**, 331 mg, 0.451 mmol) in 20 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was

purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13n** as a white solid (147.5 mg, 47%).

³¹P NMR (202 MHz, MeOD) δ 5.71, 5.09.

¹H NMR (500 MHz, MeOD) δ 8.20-8.18 (m, 1H, H₈ naph), 7.90, 7.88 (2s, 1H, H₈), 7.83- 7.78 (m, 1H, H₅-Naph), 7.65, 7.61 (2d, J=8.5 Hz, 1H, H₄-Naph), 7.53–7.44 (m, 3H, H₇, H₆, H₂-Naph), 7.37-7.30 (m, 1H, H₃-Naph), 7.23–7.18 (m, 5H, OCH₂Ph), 5.96, 5.93 (2s, 1H, H_{1'}), 5.08-4.99 (m, 2H, OCH₂Ph), 4.62-4.57 (m, 2H, H_{5'}), 4.34-4.23 (m, 2H, H_{3'}, H_{4'}), 3.81, 3.76 (2d, J= 6.0 Hz, 1H, CHCH(CH₃)₂), 2.00–1.92 (m, 1H, CHCH(CH₃)₂), 1.00, 0.96 (2s, 3H, 2'CCH₃), 0.80- 0.74 (m, 6H, CHCH(CH₃)₂).

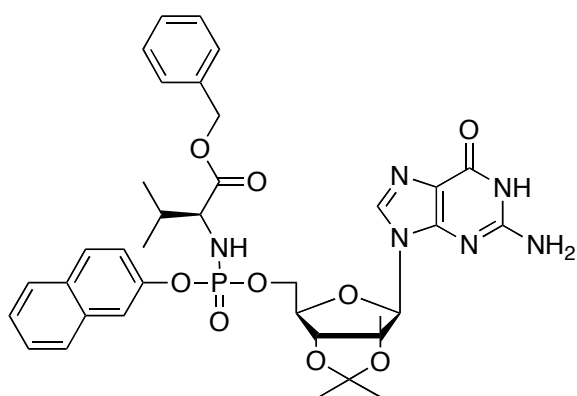
¹³C NMR (126 MHz, MeOD) δ 174.16, 137.97 (2d, ³J_{C-C-N-P} = 2.5 Hz, C=O), 159.58 (C6), 155.29, 155.25 (C2), 152.63 (C4), 148.01, 147.94 (ipso Naph), 138.13, 137.72 (C8), 137.01, 136.92 (OCH₂Ph), 136.25, 136.22 (C10-Naph), 129.51, 129.48, 129.32, 129.24, 128.84 (CH-Naph and OCH₂Ph), 127.94, 127.89 (C9-Naph), 127.80, 127.77, 127.63, 127.49, 126.51, 126.49, 126.06, 125.92, 122.92, 122.78 (CH-Naph and OCH₂Ph), 118.08 (C5), 116.46, 116.00 (2d, ³J_{C-C-O-P} = 3.0 Hz, C2-Naph), 93.26, 93.10 (C1'), 82.27, 82.13 (2d, ³J_{C-C-O-P} = 8.5 Hz, C4'), 80.02 (C2'), 74.79, 74.47 (C3'), 67.93, 67.91 (OCH₂Ph), 67.55, 67.49 (2d, ²J_{C-O-P} = 4.5 Hz, C5'), 61.91 (CHCH(CH₃)₂), 33.28, 33.14 (2d, ³J_{C-C-N-P} = 7.5 Hz, CHCH(CH₃)₂), 20.50, 20.48 (2'CCH₃), 19.52, 19.41, 18.39, 18.31 (CHCH(CH₃)₂).

HPLC (System 1) t_R = 17.11, 17.71 min.

HRMS calculated for $C_{33}H_{37}N_6O_9PNa$: 715.2244; found 715.2257.

CHN calculated for $C_{33}H_{37}N_6O_9P + 1.0 H_2O$: C 55.77, H 5.53, N 11.83; found: C 55.95, H 5.44, N 11.49.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[β -naphthyl-(benzoxy-L-valinyl)] phosphate (4.12o**).**



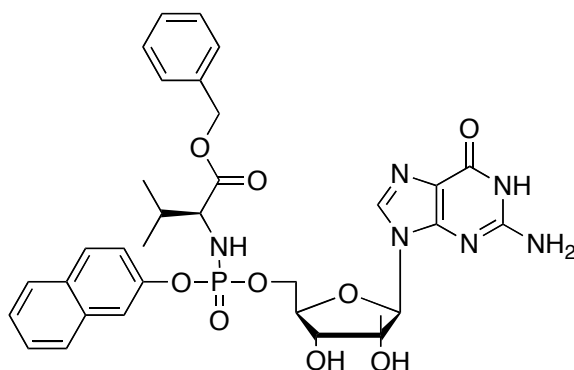
Prepared according to the Standard Procedure 4 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine (**4.11**, 250 mg, 0.741 mmol), β -naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3u**, 640 mg, 1.48 mmol), *t*BuMgCl (1.48

ml, 1.48 mmol) in 4 ml of dry THF. The crude mixture was purified by column chromatography, using $CHCl_3/MeOH$ (2 to 5%, gradient) as an eluent, to give a pure product **4.12o** as white foam (234 mg, 43%).

^{31}P NMR (202 MHz, MeOD) δ 5.07, 5.05.

1H NMR (500 MHz, MeOD) δ 7.88–7.80 (m, 3H, Naph), 7.74, 7.71 (2s, 1H, H_8) 7.51–7.44 (m, 2H, Naph), 7.39–7.34 (m, 2H, Naph), 7.33–7.27 (m, 5H, OCH_2Ph), 6.10, 6.09 (2s, 1H, $H_{1'}$), 5.15, 5.11, 5.02, 4.97 (2AB, J_{AB} = 10.0 Hz, 2H, OCH_2Ph) 4.67–4.52 (m, 2H, $H_{3'}$, $H_{5'a}$), 4.44–4.38 (m, 2H, $H_{5'b}$, $H_{4'}$), 3.85–3.75 (m, 1H, $CHCH(CH_3)_2$), 2.12–2.01 (m, 1H, $CHCH(CH_3)_2$), 1.59, 1.57, 1.36, 1.35 (4s, 6H, 2x CH_3 isopropylidene), 1.16, 1.02 (2s, 3H, $2'CCH_3$) 0.92–0.86 (m, 6H, $CHCH(CH_3)_2$).

Synthesis of 2'-C- β -methylguanosine 5'-O-[β -NaphThyl-(benzoxy-L-valinyl)] phosphate (4.13o).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[β -Naphthyl-(benzoxy-L-valinyl)] phosphate (4.12o, 234 mg, 0.319 mmol) in 10 ml

of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product 4.13o as a white solid (32.8 mg, 15%).

³¹P NMR (202 MHz, MeOD) δ 5.03, 4.90.

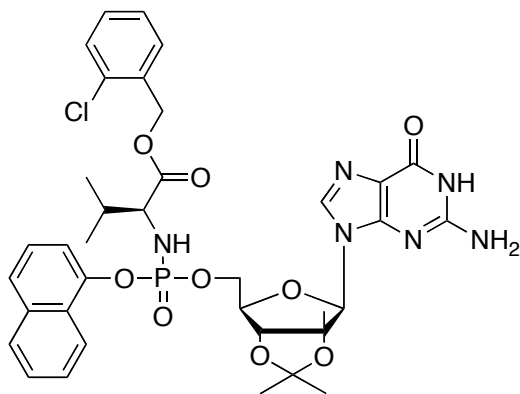
¹H NMR (500 MHz, MeOD) δ 7.87-7.81 (m, 3H, CH-Naph), 7.77- 7.70 (m, 2H, CH-Naph and H₈), 7.53-7.49 (m, 3H, CH-Naph), 7.30-7.20 (m, 5H, OCH₂Ph), 5.92, 5.91 (2s, 1H, H_{1'}), 5.09, 5.05, 4.94, 4.88 (2AB, *J*_{AB} = 12.0 Hz, OCH₂Ph), 4.60-4.47 (m, 2H, H_{5'}), 4.30-4.09 (m, 2H, H_{3'}, H_{4'}), 3.81-3.76 (m, 1H, CHCH(CH₃)₂), 2.06 – 1.99 (m, 1H, CHCH(CH₃)₂), 1.00, 0.99 (2s, 3H, 2'CCH₃), 0.89-0.82 (m, 6H, CHCH(CH₃)₂).

¹³C NMR (126 MHz, MeOD) δ 174.01, 173.79 (2d, ³*J*_{C-C-N-P} = 2.8 Hz, C=O), 159.42, 159.40 (C6), 155.30, 155.28 (C2), 149.80, 149.74 (C4), 149.71, 149.66 (ipso Naph), 137.85, 137.56 (C8), 137.15, 137.03 (ipso OCH₂Ph), 135.30, 135.26 (C9-Naph), 132.40, 132.38 (C10-Naph), 130.89, 130.84, 129.53, 129.52, 129.47, 129.41, 129.31, 129.27, 128.77, 128.74, 128.54, 128.48, 127.77, 126.55, 121.52 (CH-Naph and OCH₂Ph), 118.03 (C5), 117.88, 117.85 (CH-Naph), 93.18, 92.91 (C1'), 82.21, 82.04 (2d, ³*J*_{C-C-O-P} = 8.5 Hz, C4'), 79.99, 79.96 (C2'), 74.65, 74.23 (C3'), 67.90, 87.86 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 67.86, 67.76 (OCH₂Ph), 62.07, 61.99 (CHCH(CH₃)₂), 33.31 (2d, ³*J*_{C-C-N-P} = 7.5 Hz, CHCH(CH₃)₂), 20.32, 20.27 (2'CCH₃), 19.52, 19.47, 18.38, 18.21 (CHCH(CH₃)₂).

HPLC (System 1) *t*_R = 17.28, 17.92 min.

HRMS calculated for C₃₃H₃₇N₆O₉PNa: 715.2257; found 715.2266.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(*o*-chloro-benzoxy-L-valinyl)] phosphate (4.12p).



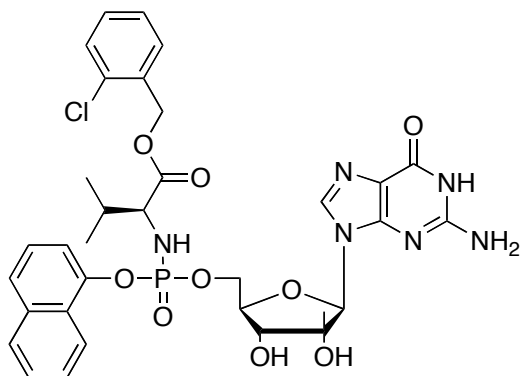
Prepared according to the Standard Procedure 4 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine (**4.11**, 250 mg, 0.741 mmol), α -naphthyl-(*o*-chloro-benzoxy-L-valinyl) phosphorochloridate (691 mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in

4 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as en eluent, to give a pure product **4.12p** as white foam (367 mg, 51%).

³¹P NMR (202 MHz, MeOD) δ 5.34, 5.23.

¹H NMR (500 MHz, MeOD) δ 8.19-8.16 (m, 1H, H₈-Naph), 7.88-7.83 (m, 1H, H₅-Naph), 7.76, 7.73 (2s, 1H, H₈), 7.72-7.67 (m, 1H, H₄-Naph), 7.56-7.52 (m, 3H, H₇, H₆, H₂-naph), 7.43-7.19 (m, 6H, H₃-Naph and OCH₂Ph), 6.05, 6.04 (2s, 1H, H_{1'}), 5.20, 5.17, 5.13, 5.11 (2AB, *J*_{AB}= 10.5 Hz, 2H, OCH₂Ph), 4.62-4.53 (m, 2H, H_{3'} and H_{5'a}), 4.48-4.36 (m, 2H, H_{5'b} and H_{4'}), 3.88, 3.82 (2d, *J*= 6.0 Hz, CHCH(CH₃)₂), 2.15 -2.02 (m, 1H, CHCH(CH₃)₂) 1.57, 1.38 (2s, 6H, 2x CH₃ isopropylidene) 1.04, 0.96 (2s, 3H, 2'CCH₃), 0.91, 0.88 (2d, *J*= 7.5 Hz, 6H, CHCH(CH₃)₂).

Synthesis of 2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(*o*-chloro-benzoxy-L-valinyl)] phosphate (4.13p).



Prepared according to the Standard Procedure 5 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(*o*-chloro-benzoxy-L-valinyl)] phosphate (**4.12p**, 367 mg, 0.478 mmol) in 20 ml of 60% CH₃COOH in water

at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13p** as a white solid (112.4 mg, 32%).

³¹P NMR (202 MHz, MeOD) δ 5.10, 4.91.

¹H NMR (500 MHz, MeOD) δ 8.18-8.16 (m, 1H, H₈-Naph), 7.89-7.84 (m, 2H, H₈ and H₅-Naph), 7.69, 7.67 (2d, *J* = 8.5 Hz, 1H, H₄-Naph), 7.53-7.18 (m, 8H, H₇, H₆, H₂, H₃-Naph and OCH₂Ph), 5.92, 5.91 (2s, 1H, H_{1'}), 5.13-4.98 (m, 2H, OCH₂Ph), 4.59- 4.56 (m, 2H, H_{5'}), 4.31-4.19 (m, 2H, H_{3'} and H_{4'}), 3.83, 3.80 (2d, *J* = 6.0 Hz, CHCH(CH₃)₂), 2.07-1.99 (m, 4H, CHCH(CH₃)₂ and 2'CCH₃), 0.87-0.82 (m, 6H, CHCH(CH₃)₂).

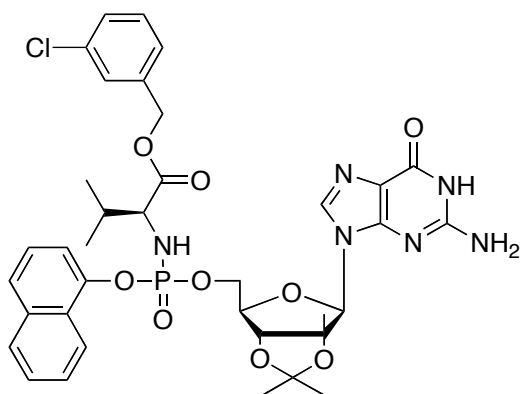
¹³C NMR (126 MHz, MeOD) δ 173.85, 173.61 (C=O), 159.45 (C6), 155.25 (C2), 152.58 (C4), 148.00, 147.93 (ipso Naph), 137.98, 137.76 (C8), 136.26, 136.21 (C10-Naph), 134.67 (ipso OCH₂(*o*-Cl)Ph), 134.45 (C-Cl OCH₂(*o*-Cl)Ph), 131.54, 131.50, 130.92, 130.44, 128.83, 128.76, 128.15 (OCH₂(*o*-Cl)Ph and CH-Naph), 127.86 (C9-Naph), 127.73, 127.46, 126.50, 126.42, 125.93, 122.83, 122.80 (OCH₂(*o*-Cl)Ph and CH-Naph), 118.12, 118.10 (C5), 116.17 (C2-Naph), 93.35, 93.22 (C1'), 82.30, 82.15 (2d, ³*J*_{C-C-O-P} = 8.5 Hz, C4'), 79.93 (C2'), 74.83, 74.64 (C3'), 68.31 (d, ²*J*_{C-O-P} = 3.5 Hz, C5'), 67.74 (d, ²*J*_{C-O-P} = 4.2 Hz, C5'), 65.10 (OCH₂(*o*-Cl)Ph), 62.08, 62.02 (CHCH(CH₃)₂), 33.24, 33.07 (d, ³*J*_{C-C-N-P} = 5.0 Hz, CHCH(CH₃)₂), 20.39, 2.38 (2'CCH₃), 19.50, 19.36, 18.37, 18.24 (CHCH(CH₃)₂).

HPLC (System 1) t_R = 18.75, 19.01 min.

HRMS calculated for C₃₃H₃₇N₆O₉PClNa: 749.1868; found 749.1879.

CHN calculated for C₃₃H₃₇N₆O₉PCl + 1.0 H₂O: C 53.19, H 5.14, N 11.28; found: C 53.20, H 4.90, N 11.04.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine 5'-*O*-[α-naphthyl-(*m*-chloro-benzoxo-L-valinyl)] phosphate (4.12r**).**



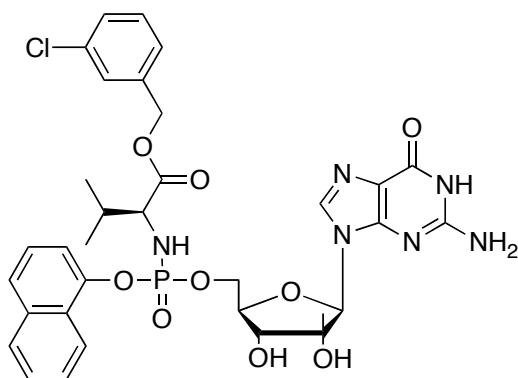
Prepared according to the Standard Procedure 4 from: 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine (**4.11**, 250 mg, 0.741 mmol), α-naphthyl-(*m*-chloro-benzoxo-L-valinyl)

phosphorochloridate (691 mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in 4 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient), to give a pure product **4.12r** as white foam (288 mg, 51%).

³¹P NMR (202 MHz, MeOD) δ 5.27, 5.11, 4.92, 4.87.

¹H NMR (500 MHz, MeOD) δ 8.19-8.13 (m, 1H, H₈-Naph), 7.89-7.84 (m, 1H, H₅-Naph), 7.75-7.66 (m, 2H, H₈ and H₄-Naph), 7.54-7.50 (m, 3H, H₇, H₆, H₂-Naph), 7.43-7.31 (m, 2H, H₃-Naph and OCH₂(*m*-Cl)Ph), 7.26-7.21 (m, 3H, OCH₂(*m*-Cl)Ph), 6.05, 6.04 (2s, 1H, H_{1'}), 5.10-5.02 (m, 2H, OCH₂(*m*-Cl)Ph), 4.61-4.35 (m, 4H, H_{5'}, H_{4'} and H_{3'}), 3.88-3.79 (m, 1H, CHCH(CH₃)₂), 2.13-2.01 (m, 1H, CHCH(CH₃)₂), 1.59, 1.57, 1.38, 1.37 (4s, 6H, 2x CH₃ isopropylidene) 1.04, 0.99, 0.95, 0.96 (4s, 3H, 2'CCH₃), 0.92-0.86 (m, 6H, CHCH(CH₃)₂).

Synthesis of 2'-C-β-methylguanosine 5'-O-[α-naphthyl-(*m*-chloro-benzoxo-L-valinyl)] phosphate (4.13r).



Prepared according to the Standard Procedure 5 from: 2', 3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(*m*-chloro-benzoxo-L-valinyl)] phosphate (**4.12r**, 288 mg, 0.375 mmol) in 20 ml of 60% CH₃COOH in water at 90 °C overnight. The crude

mixture was purified by column chromatography, using CHCl₃/MeOH (9:1), to give a pure product **4.13r** as a white solid (97mg, 36%).

³¹P NMR (202 MHz, MeOD) δ 5.27, 5.11, 4.92, 4.87.

¹H NMR (500 MHz, MeOD) δ 8.19-8.15 (m, 1H, H₈-Naph), 7.83-7.77 (m, 1H, H₅-Naph), 7.66, 7.64, 7.62, 7.60 (4s, 1H, H₈), 7.53-7.50 (m, 1H, H₄-Naph), 7.47-7.10 (m, 8H, H₇, H₆, H₂, H₃-Naph, OCH₂(*m*-Cl)Ph), 5.95, 5.94, 5.93, 5.92 (4s, 1H, H_{1'}), 5.04-4.90 (m, 2H, OCH₂(*m*-Cl)Ph), 4.61-4.55 (m, 2H, H_{5'}), 4.35-4.20 (m, 2H, H_{3'},

and H₄), 3.83- 3.75 (m, 1H, CHCH(CH₃)₂), 2.03 – 1.93 (m, 1H, CHCH(CH₃)₂), 1.00, 0.96, 0.95 (3s, 3H, 2'CCH₃), 0.84–0.75 (m, 6H, CHCH(CH₃)₂).

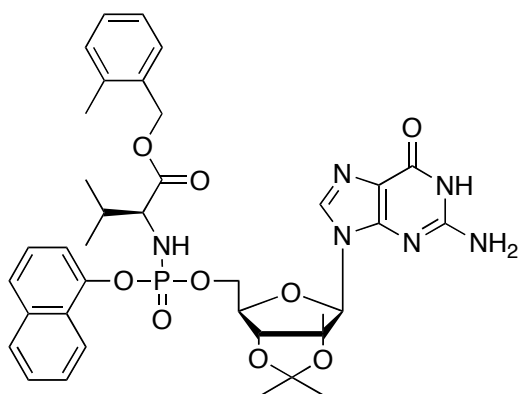
¹³C NMR (126 MHz, MeOD) δ 174.04, 173.95 (2d, ³J_{C-C-N-P} = 2.5 Hz, C=O), 173.86, 173.70 (2d, ³J_{C-C-N-P} = 2.7 Hz, C=O), 159.60, 159.57 (C6), 155.30, 155.26 (C2), 152.62, 152.60 (C4), 147.96, 147.94 (2d, ²J_{C-O-P} = 7.0 Hz, ipso Naph), 139.26, 139.23, 139.16 (C-Cl OCH₂(*m*-Cl)Ph), 138.10, 138.00, 137.78, 137.67 (C8), 136.23, 136.20, 136.18 (C10-Naph), 135.28, 135.25 (ipso OCH₂(*m*-Cl)Ph), 131.06, 131.04, 129.30, 129.28, 129.26, 129.25, 129.19, 129.08, 128.85, 128.84, 128.78 (CH-Naph and OCH₂(*m*-Cl)Ph), 127.88 (C9-Naph), 127.80, 127.76, 127.65, 127.62, 127.55, 127.50, 127.44, 126.52, 126.46, 126.06, 125.95, 122.89, 122.84, 122.80, 122.76 (CH-Naph and OCH₂(*m*-Cl)Ph), 118.10 (C5), 116.46 (d, ³J_{C-C-O-P} = 2.5 Hz, C2-Naph), 116.24 (d, ³J_{C-C-O-P} = 4.0 Hz, C2-Naph), 116.21 (d, ³J_{C-C-O-P} = 3.3 Hz, C2-Naph), 116.02 (d, ³J_{C-C-O-P} = 3.0 Hz, C2-Naph), 93.30, 93.18, 93.08 (C1'), 82.26, 82.16, 82.10, 80.00 (4d, ³J_{C-C-O-P} = 5.0 Hz, C4'), 79.97 (C2'), 74.85, 74.79, 74.66, 74.43 (C3'), 67.97, 67.74, 67.68, 67.51 (4d, ²J_{C-O-P} = 5.0 Hz, C5'), 66.98, 66.95, 66.90, 66.86 (OCH₂(*m*-Cl)Ph), 62.02, 61.98, 61.89 (CHCH(CH₃)₂), 33.28, 33.23, 33.15, 33.08 (4d, ³J_{C-C-O-P} = 2.5 Hz, CHCH(CH₃)₂), 20.50 (2'CCH₃), 19.55, 19.42, 18.43, 18.40, (CHCH(CH₃)₂).

HPLC (System 1) t_R = 18.91, 19.15, 19.31 min.

HRMS calculated for C₃₃H₃₇N₆O₉PClNa: 749.1868; found 749.1881.

CHN calculated for C₃₃H₃₇N₆O₉PCl + 1.0 H₂O: C 53.19, H 5.14, N 11.28; found C 53.25, H. 5.28, N 11.25.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine 5'-*O*-[α-naphthyl-(*o*-methyl-benzoxy-L-valinyl)] phosphate (4.12s).



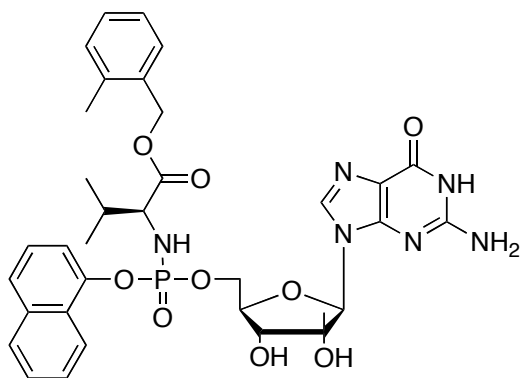
Prepared according to the Standard Procedure 4 from: 2'3'-*O,O*-isopropylidene-2'-*C*-β-methylguanosine (4.11, 250 mg, 0.741 mmol), α-naphthyl-(*o*-methyl-benzoxy-L-valinyl) phosphorochloridate (618 mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in

4 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (2 to 8%, gradient), to give a pure product as white foam (172 mg, 31%).

^{31}P NMR (202 MHz, MeOD) δ 5.11, 4.97.

^1H NMR (500 MHz, MeOD) δ 8.19–8.15 (m, 1H, $\text{H}_8\text{-Naph}$), 7.89–7.85 (m, 1H, $\text{H}_5\text{-Naph}$), 7.75–7.68 (m, 2H, H_8 and $\text{H}_4\text{-Naph}$), 7.54–7.51 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.43–7.37 (m, 1H, $\text{H}_3\text{-Naph}$), 7.20–7.09 (4H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 6.06, 6.05 (2s, 1H, $\text{H}_{1'}$), 5.16–5.08 (m, 2H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 4.60, 4.50 (2d, $J=3.5$ Hz, 1H, $\text{H}_{3'}$), 4.42–4.28 (m, 3H, $\text{H}_{5'}$ and $\text{H}_{4'}$), 3.85, 3.78 (2d, $J=6.5$ Hz, 1H, $\text{CHCH}(\text{CH}_3)_2$), 2.31 (s, 3H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 2.11–2.00 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.59, 1.57, 1.38, 1.37 (4s, 6H, 2 x CH_3 isopropylidene) 1.02, 0.94 (2s, 3H, $2'\text{-CCH}_3$), 0.89–0.85 (m, 6H, $\text{CHCH}(\text{CH}_3)_2$).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(*o*-methyl-benzoxo-L-valinyl)] phosphate (4.13s).



Prepared according to the Standard Procedure 5 from: 2',3'-*O,O*-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -Naphthyl-(*o*-methyl-benzoxo-L-valinyl)] phosphate (**4.12s**, 172 mg, 0.230 mmol) in 10 ml of 60% CH_3COOH in

water at 90 °C overnight. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (9:1), to give a pure product **4.13s** as a white solid (90 mg, 55%).

^{31}P NMR (202 MHz, MeOD) δ 5.11, 4.97.

^1H NMR (500 MHz, MeOD) δ 8.17 (d, $J=8.0$ Hz, 1H, $\text{H}_8\text{-Naph}$), 7.85, 7.83 (2s, 1H, H_8), 7.81 (d, 1H, $J=7.5$ Hz, $\text{H}_5\text{-Naph}$), 7.66, 7.63 (2d, $J=8.00$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.52–7.44 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.36–7.32 (m, 1H, $\text{H}_3\text{-Naph}$), 7.20–7.14 (m, 2H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 7.11–7.05 (m, 2H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 5.93, 5.92 (2s, 1H, $\text{H}_{1'}$), 5.06–4.97 (m, 2H, $\text{OCH}_2(o\text{-CH}_3)\text{Ph}$), 4.60–4.54 (m, 2H, $\text{H}_{5'}$), 4.33–4.20 (m, 2H, $\text{H}_{3'}$ and

H₄), 3.79, 3.76 (2d, $J = 6.0$ Hz, 1H, CHCH(CH₃)₂), 2.24, 2.23 (2s, 3H, OCH₂(*o*-CH₃)Ph), 2.05–1.92 (m, 1H, CHCH(CH₃)₂), 1.00, 0.95 (2s, 3H, 2'CCH₃), 0.83–0.77 (m, 6H, CHCH(CH₃)₂).

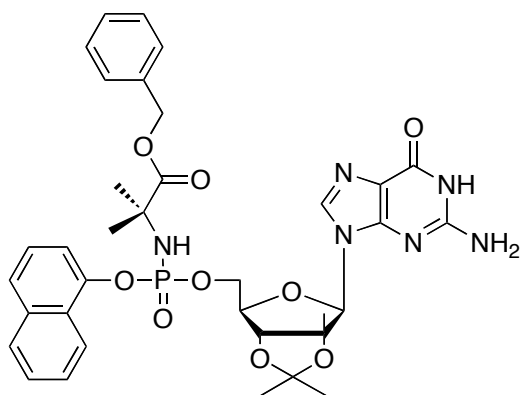
¹³C NMR (126 MHz, MeOD) δ 174.06 (d, $^3J_{C-C-N-P} = 2.7$ Hz, C=O), 173.81 (d, $^3J_{C-C-N-P} = 3.1$ Hz, C=O), 159.49, 159.46 (C6), 155.25, 155.21 (C2), 152.59 (C4), 148.01, 147.96 (ipso Naph), 138.22 (C-CH₃ OCH₂(*o*-CH₃)Ph), 138.01, 137.77 (C8), 136.26, 136.21 (C10-Naph), 134.84, 134.82 (ipso OCH₂(*o*-CH₃)Ph), 131.25, 130.63, 130.60, 129.65, 129.64, 128.83, 128.76 (CH-Naph and OCH₂(*o*-CH₃)Ph), 127.89, 127.84 (C9-Naph), 127.74, 127.46, 126.97, 126.52, 126.43, 125.92, 122.87, 122.82 (CH-Naph and OCH₂(*o*-CH₃)Ph), 118.12, 118.10 (C5), 116.23 (d, $^3J_{C-C-O-P} = 2.6$ Hz, C2-Naph), 116.15 (d, $^3J_{C-C-O-P} = 3.1$ Hz, C2-Naph), 93.33, 93.20 (C1'), 82.30 (d, $^3J_{C-C-O-P} = 7.9$ Hz, C4'), 82.15 (d, $^3J_{C-C-O-P} = 8.3$ Hz, C4'), 79.96, 79.93 (C2'), 74.84, 74.64 (C3'), 68.29, 67.70 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 66.23 (OCH₂(*o*-CH₃)Ph), 62.09 (CHCH(CH₃)₂), 33.30 (d, $^3J_{C-C-N-P} = 7.0$ Hz, CHCH(CH₃)₂), 33.10 (d, $^3J_{C-C-N-P} = 7.4$ Hz, CHCH(CH₃)₂), 20.42, 19.48 (2'CCH₃), 18.38, 18.24 (OCH₂(*o*-CH₃)Ph).

HPLC (System 1) $t_R = 18.59, 18.75$ min.

HRMS C₃₄H₃₉N₆O₉PClNa: found 729.2407, calc. 729.2414

CHN calculated for C₃₄H₃₉N₆O₉PCl + 1.0 H₂O: C56.75, H5.70, N11.60; found: C 56.33, H 5.82, N 11.20.

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(benzoxy-dimethylglycinyl)] phosphate (4.12t).



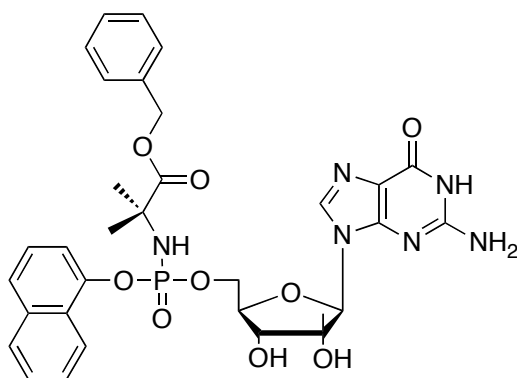
Prepared according to the Standard Procedure 4 from: 2'3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine (**4.11**, 250 mg, 0.741 mmol), α -Naphthyl-(benzoxy-dimethylglycinyl) phosphorochloridate (**3.3p**, 619 mg, 1.48 mmol), *t*BuMgCl (1.48 ml, 1.48 mmol) in

4 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient), to give a pure product **4.12t** as white foam (140 mg, 39%).

^{31}P NMR (202 MHz, CDCl_3) δ 2.75, 2.28.

^1H NMR (500 MHz, CDCl_3) δ 8.19–8.11 (m, 1H, $\text{H}_8\text{-Naph}$), 7.84–.68 (m, 2H, $\text{H}_5\text{-Naph}$ and H_8), 7.58–7.43 (m, 4H, H_4 , H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.41–7.38 (m, 1H, $\text{H}_3\text{-Naph}$), 7.33 – 7.29 (m, 5H, OCH_2Ph), 6.25 (bs, 1H, NH), 6.00 (s, 1H, $\text{H}_{1'}$), 5.19–5.13 (m, 2H, CH_2 ester), 4.80–4.68 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{5'a}$), 4.39–4.30 (m, 2H, $\text{H}_{5'b}$ and $\text{H}_{4'}$), 1.65–1.57 (m, 9H, CH_3 isopropylidene and $\text{C}(\text{CH}_3)_2$), 1.39 (s, 3H, CH_3 isopropylidene), 1.16 (s, 3H, $2'\text{CCH}_3$).

Synthesis of 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(benzoxy-dimethylglycyl)] phosphate (4.12t).



Prepared according to the Standard Procedure 5 from: 2',3'-*O,O*-isopropylidene-2'-*C*- β -methylguanosine 5'-*O*-[α -naphthyl-(benzoxy-dimethylglycyl)] phosphate (**4.12t**, 140 mg, 0.292 mmol) in 10 ml of 60% CH_3COOH in water at 90 °C overnight.

The crude mixture was purified by column chromatography $\text{CHCl}_3/\text{MeOH}$ (9:1) to give a pure product **4.13t** as a white solid (51 mg, 39%).

^{31}P NMR (202 MHz, MeOD) δ 2.75, 2.62.

^1H NMR (500 MHz, MeOD) δ 8.20–8.18 (m, 1H, $\text{H}_8\text{-Naph}$), 7.87 – 7.82 (m, 2H, $\text{H}_5\text{-Naph}$ and H_8), 7.68 – 7.63 (m, 1H, $\text{H}_4\text{-Naph}$), 7.53–7.35 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.32–7.26 (m, 5H, OCH_2Ph), 5.94, 5.90 (2s, 1H, $\text{H}_{1'}$), 5.14, 5.11, 5.08, 5.05 (2AB, J = 10.5 Hz, 2H, OCH_2Ph), 4.60–4.49 (m, 2H, $\text{H}_{5'}$), 4.28–4.18 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{4'}$), 1.52–1.49 (m, 6H, $\text{C}(\text{CH}_3)_2$), 1.00, 0.96 (2s, 3H, $2'\text{CCH}_3$).

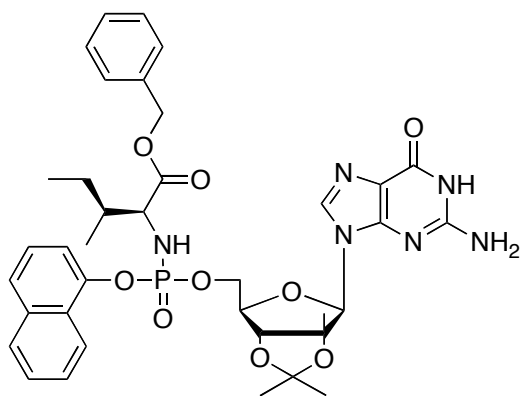
^{13}C NMR (126 MHz, MeOD) δ 176.58, 176.54 (2d, $^3J_{\text{C-C-N-P}}$ = 3.8 Hz, C=O), 159.45, 159.43 (C6), 155.29, 155.23 (C2), 152.65, 152.61 (C4), 148.07, 148.01 (2d, $^2J_{\text{C-O-P}}$ = 4.2 Hz, ipso Naph), 137.96, 137.90 (C8), 137.27, 137.25 (ipso OCH_2Ph), 136.26, 136.22 (C10-Naph), 129.57, 129.56, 129.25, 129.20, 128.82, 128.78 (CH-Naph and OCH_2Ph), 127.96, 127.91 (C9-Naph), 127.70, 127.36, 126.51, 126.44, 125.84,

125.79, 123.01 (CH-Naph and OCH₂Ph), 118.04 (C5), 116.38, 116.27 (2d, ³J_{C-C-O-P} = 3.1 Hz, C2-Naph), 93.19 (C1'), 82.24, 82.12 (2d, ³J_{C-C-O-P} = 8.5 Hz, C4'), 80.02, 79.96 (C2'), 74.72, 74.61 (C3'), 68.30, 68.26 (OCH₂Ph), 67.77, 67.56 (2d, ²J_{C-O-P} = 5.0 Hz, C5'), 58.23, 58.15, (2d, ²J_{C-N-P} = 3.7 Hz, C(CH₃)₂), 30.48, 27.82, 27.68, 27.65 (4d, ³J_{C-C-N-P} = 5.0 Hz, C(CH₃)₂), 20.46 (2'CCH₃).

HPLC (System 1) t_R = 16.83, 17.16 min.

HRMS calculated for C₃₂H₃₅N₆O₉PClNa: 701.2101; found 701.2097.

Synthesis of 2',3'-O,O-isopropylidene-2'-C-β-methylguanosine 5'-O-[α-naphthyl-(benzoxy-L-isoleuciny)] phosphate (4.12u).



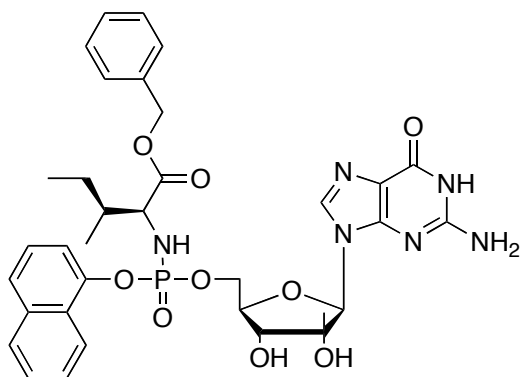
Prepared according to the Standard Procedure 4 from: 2'3'-O,O-isopropylidene-2'-C-β-methylguanosine (**4.11**, 200 mg, 0.593 mmol), α-naphthyl-(benzoxy-L-isoleuciny) phosphorochloridate (264 mg, 1.18 mmol), *t*BuMgCl (1.18 ml, 1.18 mmol) in 4 ml of dry THF. The crude mixture was

purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as eluent, to give a pure product **4.12u** as white foam (205 mg, 50%).

³¹P NMR (202 MHz, MeOD) δ 5.27, 5.08.

¹H NMR (500 MHz, MeOD) δ 8.19-8.16 (m, 1H, H₈-Naph), 7.88-7.84 (m, 1H, H₅-Naph), 7.77, 7.73 (2s, 1H, H₈) 7.73-7.67 (m, 1H, H₄-Naph), 7.55-7.50 (m, 3H, H₇, H₆, H₂-Naph), 7.43-7.37 (m, 1H, H₃-Naph), 7.33-7.27 (m, 5H, OCH₂Ph), 6.06, 6.05 (2s, 1H, H1'), 5.14-5.01 (m, 2H, OCH₂Ph), 4.60-4.42 (m, 2H, H_{3'}, H_{5'a}), 4.39- .31(m, 2H, H_{5'b}, H_{4'}) 3.92-3.85 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.81-1.73 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.64-1.63 (m, 2H, CHCH(CH₃)CH₂CH₃), 1.59, 1.57 (2s, 3H, CH₃ isopropylidene), 1.38, 1.37 (2s, 3H, CH₃ isopropylidene), 1.03, 0.96 (2s, 3H, 2'CCH₃), 0.85-0.77(m, 6H, CHCH(CH₃)CH₂CH₃ and CHCH(CH₃)CH₂CH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -naphthyl-(benzoxy-L-isoluciny)] phosphate (4.13u).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(benzoxy-L-leuciny)] phosphate (**4.12u**, 205 mg, 0.274 mmol) in

10 ml of 60% CH₃COOH in water at 90 °C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as an eluent, to give a pure product **4.13u** as a white solid (27 mg, 14%).

³¹P NMR (202 MHz, MeOD) δ 4.99, 4.90.

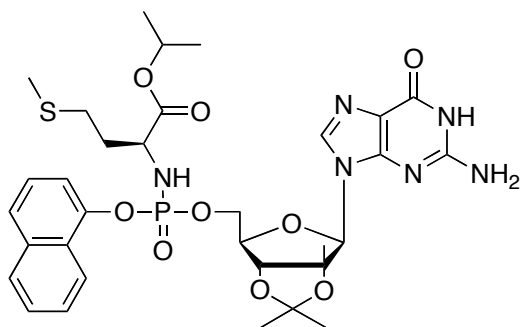
¹H NMR (500 MHz, MeOD) δ 8.18 (d, J = 7.0 Hz, 1H, H₈-Naph), 7.90–7.87 (m, 1H, H₅-Naph), 7.84, 7.83 (2s, 1H, H₈), 7.71–7.67 (m, 1H, H₄-Naph), 7.55–7.49 (m, 3H, H₇, H₆, H₂-Naph), 7.40–7.35 (m, 1H, H₃-Naph), 7.28–7.27 (m, 5H, OCH₂Ph), 5.02 (s, 1H, H_{1'}), 5.06–4.91 (m, 2H, OCH₂Ph), 4.57–4.55 (m, 2H, H_{5'}), 4.29–4.20 (m, 2H, H_{3'}, H_{4'}), 3.87–3.83 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.74–1.69 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.42–1.36 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.09–1.03 (m, 1H, CHCH(CH₃)CH₂CH₃), 1.00, 0.97 (2s, 3H, 2'CCH₃), 0.81–0.74 (m, 6H, 3H, CHCH(CH₃)CH₂CH₃ and CHCH(CH₃)CH₂CH₃).

HPLC (System 1) t_R = 18.23 min.

HRMS calculated for C₃₄H₃₉N₆O₉PNa: 729.2497; found 729.2453.

CHN calculated for C₃₄H₃₉N₆O₉PCl + 0.75H₂O: C 56.7, H 5.67, N 11.67; found: C 56.64, H 5.58, N 11.59.

Synthesis of 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -naphthyl-(isopropoxy-L-methioninyl)] phosphate (4.12v).



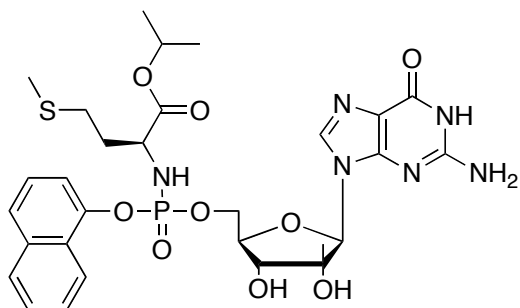
Prepared according to the Standard Procedure 4 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine (**4.11**, 250 mg, 0.741 mmol), α -naphthyl-(isopropoxy-L-methioninyl) phosphorochloridate (**3.3x**, 422 mg, 1.48

mmol) *t*BuMgCl (1.48 ml, 1.48 mmol) in 3 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (2 to 8%, gradient) as en eluent, to give a pure product **4.12v** as a white foam (314.2 mg, 59%).

³¹P NMR (202 MHz, MeOD) δ 4.80, 4.71.

¹H NMR (500 MHz, MeOD) δ 8.24–8.16 (m, 1H, H₈-Naph), 7.89–7.87 (m, 1H, H₅-Naph), 7.87, 7.82 (s, 1H, H₈), 7.74–7.70 (m, 1H, H₄-Naph), 7.59–7.51 (m, 3H, H₇, H₆, H₂-Naph), 7.47–7.40 (m, 1H, H₃-Naph), 6.10, 6.07 (2s, 1H, H1'), 5.00–4.94 (m, 1H, OCH(CH₃)₂), 4.63–4.39 (m, 4H, H_{3'}, H_{5'}, H_{4'}), 4.15–4.09 (m, 1H, CHCH₂CH₂SCH₃), 2.51–2.38 (m, 2H, CHCH₂CH₂SCH₃), 2.05–1.90 (m, 5H, CHCH₂CH₂SCH₃ and CHCH₂CH₂SCH₃), 1.61, 1.58 (2s, 3H, CH₃ isopropylidene), 1.40, 1.39 (2s, 3H, CH₃ isopropylidene), 1.22–1.18 (m, 6H, OCH(CH₃)₂), 1.05, 1.02 (2s, 3H, 2'CCH₃).

Synthesis of 2'-C- β -methylguanosine 5'-O-[α -Nnaphthyl-(isopropoxy-L-methioninyl)] phosphate (4.13v).



Prepared according to the Standard Procedure 5 from: 2',3'-O,O-isopropylidene-2'-C- β -methylguanosine 5'-O-[α -Naphthyl-(isopropoxy-L-methioninyl)] phosphate (296.6mg, 0.414 mmol) in 20 ml of 60%CH₃COOH in

water at 90°C overnight. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (9:1) as en eluent, to give a pure product as a white solid (144.3mg, 52%).

^{31}P NMR (202 MHz, MeOD) δ 4.60, 4.57

^1H NMR (500 MHz, MeOD) δ 8.21–8.17 (m, 1H, H₈-Naph), 7.93, 7.91 (2s, 1H, H₈), 7.85–7.80 (m, 1H, H₅-Naph), 7.68–7.64 (m, 1H, H₄-Naph), 7.55–7.46 (m, 3H, H₇, H₆, H₂-Naph), 7.40–7.34 (m, 1H, H₃-Naph), 5.96, 5.95 (2s, 1H, H_{1'}), 4.90–4.85 (m, 1H, OCH(CH₃)₂), 4.71–4.59 (m, 2H, H_{5'}), 4.36–4.24 (m, 2H, H_{3'}, H_{4'}), 4.10–4.03 (m, 1H, CHCH₂CH₂SCH₃), 2.45–2.29 (m, 2H, CHCH₂CH₂SCH₃), 1.96–1.80 (m, 5H, CHCH₂CH₂SCH₃ and CHCH₂CH₂SCH₃), 1.15–1.11 (m, 6H, OCH(CH₃)₂), 1.02, 0.98 (2s, 3H, 2'CCH₃).

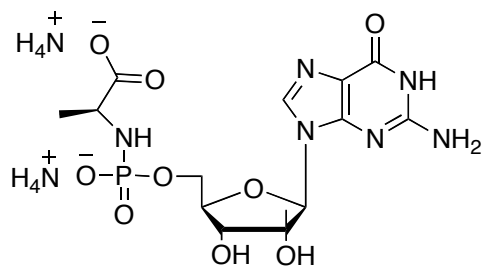
^{13}C NMR (126 MHz, MeOD) δ 173.99 (d, $^3J_{\text{C-C-N-P}} = 3.3$, C=O), 173.70 (d, $^3J_{\text{C-C-N-P}} = 4.0$ Hz, C=O), 159.56 (C6), 155.30, 155.26 (C2), 152.67 (C4), 148.00, 147.95 (2d, $^2J_{\text{C-O-P}} = 7.0$ Hz, ipso Naph), 138.02, 137.81 (C8), 136.28, 136.23 (C10-Naph), 128.87, 128.84 (CH-Naph), 127.88, 127.87 (C9-Naph), 127.79, 127.54, 127.50, 126.52, 125.98, 122.87, 122.83 (CH-Naph), 118.08 (C5), 116.29, 116.13 (2d, $^3J_{\text{C-C-O-P}} = 3.0$ Hz, C2-Naph), 93.27, 93.19 (C1'), 82.28, 82.16 (2d, $^3J_{\text{C-C-O-P}} = 8.0$ Hz, C4'), 80.06, 80.02 (C2'), 74.76, 74.64 (C3'), 70.42, 70.39 (OCH(CH₃)₂), 68.12, 67.74 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 55.32, 55.23 (CHCH₂CH₂SCH₃), 34.46 (d, $^3J_{\text{C-C-N-P}} = 6.6$ Hz, CHCH₂CH₂SCH₃), 34.23 (d, $^3J_{\text{C-C-N-P}} = 7.4$ Hz, CHCH₂CH₂SCH₃), 30.94, 30.87 (CHCH₂CH₂SCH₃), 21.98, 21.96 (OCH(CH₃)₂), 20.48 (2'CCH₃), 15.24, 15.21 (CHCH₂CH₂SCH₃)

HPLC (System 1) $t_{\text{R}} = 16.15, 16.45$ min.

HRMS calculated for C₂₉H₃₇N₆O₉PSNa: 699.1978; found. 699.1951.

CHN calculated for C₂₉H₃₇N₆O₉PS + 1.0 H₂O: C 50.14, H 5.66, N 12.10; found. C 50.07, H 5.41, N 11.86.

Synthesis of 2'-C- β -methylguanosine 5'-(L-alaninyl) phosphate diammonium salt (4.15).



135 mg of **4.13g** was dissolved in a mixture of water and triethylamine (1:1, 10.0 ml) and heated at 35 °C and stirred overnight. After that time TLC analysis indicated no traces of starting material. The reaction mixture was

evaporated to dryness and purified on silica gel, using a mixture of *i*-

propanol/water/ammonia (8:1:1) as an eluent. The pure compound was obtained as a white solid (63 mg, 62%).

^{31}P NMR (202 MHz, D_2O) δ 7.20.

^1H NMR (500 MHz, D_2O) δ 8.12 (s, 1H, H_8), 5.83 (s, 1H, $\text{H}_{1'}$), 4.19-4.07 (m, 4H, H_5' , H_4' and H_3'), 3.57 (q, $J = 7.5$ Hz, 1H, CHCH_3), 1.24 (d, $J = 7.5$ Hz, 3H, CHCH_3), 0.89 (s, 3H, $2'\text{CCH}_3$).

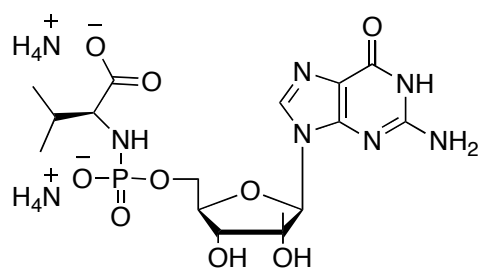
^{13}C NMR (500 MHz, D_2O) δ 182.68 (C=O), 158.79 (C6), 153.76 (C2), 151.00 (C4), 137.11 (C8), 115.93 (C5), 90.57 (C1'), 80.90 (d, $^3J_{\text{C-C-O-P}} = 8.3$ Hz, C4'), 79.34 (C2'), 71.94 (3'), 62.50 (d, $^2J_{\text{C-O-P}} = 3.7$ Hz, C5'), 52.31 (CHCH_3), 21.27 (d, $^3J_{\text{C-C-O-P}} = 3.7$ Hz, CHCH_3), 18.81 ($2'\text{CCH}_3$).

HPLC (System 1) $t_R = 1.83$ min.

MS (ES-) m/z : 446.11 (M, 100%).

HRMS calculated for $\text{C}_{14}\text{H}_{20}\text{N}_6\text{O}_9\text{P}$: 447.1029; found. 447.1011.

Synthesis of 2'-C- β -methylguanosine 5'-(L-valinyl) phosphate diammonium salt (4.16).



147 mg of **4.13m** was dissolved in a mixture of water and triethylamine (1:1, 10.0 ml) and heated at 35 °C and stirred overnight. After that time TLC analysis indicated no traces of starting material. The reaction mixture was evaporated to dryness and purified on silica gel, using a mixture of *i*-propanol/water/ammonia (8:1:1) as an eluent. The pure compound was obtained as a white solid (69 mg, 63%).

^{31}P NMR (202 MHz, D_2O) δ 7.51.

^1H NMR (500 MHz, D_2O) δ 8.07 (s, 1H, H_8), 5.82 (s, 1H, $\text{H}_{1'}$), 4.17-4.02 (m, 4H, H_5' , H_4' and H_3'), 3.48 (d, $J = 7.0$ Hz, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.72-1.86 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 0.94 (s, 3H, $2'\text{CCH}_3$), 0.87, 0.85 (2d, $J = 6.0$ Hz, 5H, $\text{CHCH}(\text{CH}_3)_2$).

^{13}C NMR (500 MHz, D_2O) δ 181.43 (C=O), 158.90 (C6), 153.87 (C2), 152.01 (C4), 137.68 (C8), 115.91 (C5), 90.59 (C1'), 81.03 (d, $^3J_{\text{C-C-O-P}} = 9.0$ Hz, C4'), 79.39 (C2'),

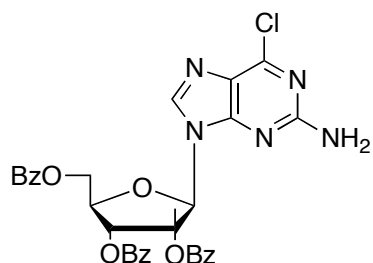
71.87 (3'), 62.36 (d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 48.89 (CHCH(CH₃)₂), 32.00 (d, $^3J_{C-C-O-P} = 7.5$ Hz, CHCH(CH₃)₂), 18.83, 18.62 (CHCH(CH₃)₂), 17.57 (2'CCH₃).

HPLC (System 1) $t_R = 1.85$ min.

MS (ES-) m : 474.15 (M-H⁺, 100%).

HRMS calculated for C₁₆H₂₄N₆O₉P: 475.1342; found. 475.1337.

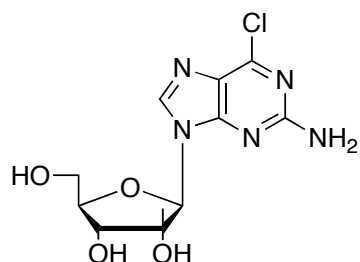
12.5 Experimental section - Chapter Five.

Synthesis of 2-amino-6-chloro-9-(2'-C-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl) purine (5.2).

To a pre-cooled (0 °C) suspension of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl- β -D-ribofuranose (10.0 g, 17.22 mmol), 2-amino-6-chloropurine (3.2 g, 18.87 mmol), and 1,8-diazabicycl[5.4.0]undec-7-ene (DBU) (7.7 ml, 51 mmol) in anhydrous acetonitrile (200 ml) trimethylsilyl triflate (12.5 ml, 68.8 mmol) was added dropwise. The reaction mixture was heated at 65°C for 4 to 6 hours, allowed to cool down to room temperature, poured into saturated aqueous sodium bicarbonate (300 ml), and extracted with dichloromethane (3x 150 ml). The combined organic phase was dried over sodium sulfate and evaporated under reduced pressure. The residue was precipitated from dichloromethane and methanol. Precipitate was filtered and washed with methanol, to give the desired compound (8.5 g, 79 %) as a white solid.

^1H NMR (500 MHz, CDCl_3) δ 8.17-8.14 (m, 2H, Bn), 8.03–7.98 (m, 4H, Bn), 8.02 (s, 1H, H₈), 7.65–7.60 (m, 1H, Bn), 7.58-7.45 (m, 3H, Bn), 7.41-7.36 (m, 4H, Bn), 6.65 (s, 1H, H_{1'}), 6.40 (d, J = 6.5 Hz, 1H, H_{3'}), 5.31 (s, 2H, NH₂), 5.08-4.82 (m, 2H, H_{5'}), 4.78- 4.75 (m, 1H, H_{4'}), 1.60 (s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, CDCl_3) δ 166.31 (C=O), 165.38 (C=O), 165.32 (C=O), 159.13 (C2), 152.87 (C6), 152.06 (C4), 141.42 (C8), 133.77, 133.69, 133.28, 129.90, 129.82 (CH Bn), 129.78 (ipso Bn), 129.70 (CH Bn), 129.41 (ipso Bn), 128.78 (ipso Bn), 128.61, 128.50, 128.41 (CH Bn), 126.00 (C5), 88.84 (C1'), 85.68 (C2'), 79.43 (C4'), 76.07 (C3'), 63.57 (C5'), 17.77 (2'CCH₃).

Synthesis of 2-amino-6-chloro-9-(2'-C-methyl- β -D-ribofuranosyl) purine (5.3).

2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl) purine (**5.2**, 2.00 g, 3.18 mmol) was suspended in dry methanol (30 ml) in sealed tube and

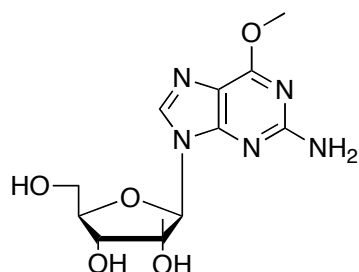
cooled down to 0 °C. After that solution was saturated with ammonia and immediately sealed. Reaction mixture was allowed to warm to ambient temperature and reaction mixture was stirred overnight. After that time solvents were evaporated under reduced pressure and crude mixture was purified on silica gel, using CHCl₃/MeOH (9:1) as eluent. Pure compound **5.3** was obtained as white solid (0.92 mg, 90%).

¹H NMR (500 MHz, DMSO) δ 8.19 (s, 1H, H₈), 7.00 (bs, 2H, NH₂), 5.85 (s, 1H, H_{1'}), 5.27 (d, *J* = 7.0 Hz, 1H, 3'OH), 5.19 (t, *J* = 5.0 Hz, 1H, 5'OH), 5.17 (s, 1H, 2'OH), 4.02 (dd, *J* = 9.0 Hz, *J* = 7.0 Hz 1H, H_{3'}), 3.91-3.81 (m, 2H, H_{4'} and H_{5'a}), 3.71- 3.68 (m, 1H, H_{5'b}), 0.83 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, DMSO) δ 159.84 (C2), 153.56 (C6), 149.51 (C4), 140.64 (C8), 123.22 (C5), 90.42 (C1'), 82.59 (C4'), 78.52 (C2'), 71.63 (C3'), 59.34 (C5'), 19.91 (2'CCH₃).

HPLC (System 1) *t*_R = 9.01 min.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**).



To a suspension of 2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-*D*-ribofuranosyl)purine (**5.2**, 3.0 g, 4.78 mmol) in anhydrous methanol (36 ml) at 0 °C NaOMe in methanol (5.4 ml, 25% w/w) was added.

The mixture was stirred at room temperature for 24 hours then quenched by addition of amberlite (H⁺). The

mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 85:15) to give the pure compound **5.4** (1.125g, 76 %) as a white solid.

¹H NMR (500 MHz, MeOD) δ 8.26 (s, 1H, H₈), 5.99 (s, 1H, H_{1'}), 4.24 (d, *J* = 9.0 Hz, 1H, H_{3'}), 4.09 (s, 3H, 6OCH₃), 4.05-4.02 (m, 2H, H_{4'} and H_{5'a}), 3.87-3.83 (m, 1H, H_{5'b}), 0.96 (s, 3H, 2'CCH₃).

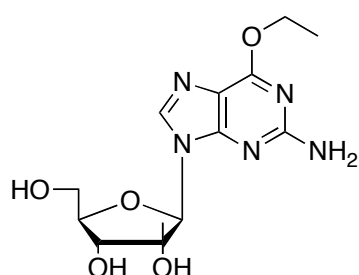
^{13}C NMR (126 MHz, MeOD) δ 162.75 (C6), 161.86 (C2), 154.50 (C4), 139.35 (C8), 115.36 (C5), 93.00 (C1'), 84.15 (C4'), 80.34 (C2'), 73.57 (C3'), 61.17 (C5'), 54.25 (6OCH₃), 20.35 (2'CCH₃).

HPLC (System 1) t_R = 8.73 min.

HRMS calculated for C₁₂H₁₇N₅O₅ Na: 334.1127, found 334.1125

CHN calculated for C₁₂H₁₇N₅O₅ + 0.75 H₂O: C 44.37, H 5.74, N 21.56; found: C 44.24, H 5.49, N 21.13.

Synthesis of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.5).



To a suspension of 2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine (**5.2**, 2.0 g, 3.19 mmol) in anhydrous ethanol (24 ml) at 0 °C, NaOEt in ethanol (1.95 g in 4.9 ml, 9 mol equivalents) was added. The mixture was stirred at 50 °C for 5 hours, cooled down to room temperature, filtrated and neutralised by addition of amberlite (H⁺). The mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 85:15) to give the pure compound (0.87 g, 84 %) as a white solid.

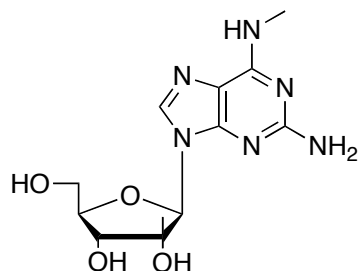
^1H NMR (500 MHz, MeOD) δ 8.25 (s, 1H, H₈), 5.99 (s, 1H, H_{1'}), 4.56 (q, J = 7.1 Hz, 2H, 6OCH₂CH₃), 4.24 (d, J = 9.1 Hz, 1H, H_{3'}), 4.05-4.02 (m, 2H, H_{4'} and H_{5'}), 3.87-3.85 (m, 1H, H_{5'}), 1.46 (t, J = 7.1 Hz, 3H, 6OCH₂CH₃), 0.96 (s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, MeOD) δ 162.42 (C6), 161.84 (C2), 154.53 (C4), 139.39 (C8), 115.65 (C5), 93.32 (C1'), 82.98 (C4'), 80.05 (C2'), 73.97 (C3'), 64.25 (6OCH₂CH₃), 61.15 (C5'), 20.35 (2'CCH₃), 14.92(6OCH₂CH₃).

HPLC (System 1) t_R = 10.55 min.

MS (TOF AP+) m/z : 326.15 (MH⁺, 100%).

HRMS Calculated for C₁₃H₁₉N₅O₅ 326.1464; found: 326.1477.

Synthesis of 2-amino-6-*N*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.6).

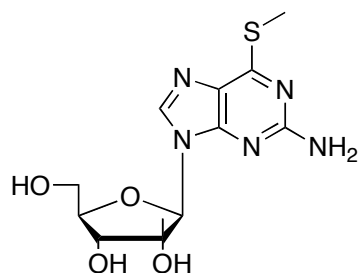
To a suspension of 2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine (**5.2**, 0.50 g, 0.80 mmol) in anhydrous methanol (10 ml) in sealed tube, was added methyl amine (4 ml, 4.8 mmol) and triethylamine (0.57 ml, 4.8 mmol). The reaction mixture was stirred at 85 °C overnight. After cooling to room temperature, solvents were evaporated and filtrated and the residue was purified by silica gel chromatography (CHCl₃/MeOH, 9:1) to give the pure compound **5.6** (0.20 g, 90 %) as a white solid.

¹H NMR (500 MHz, MeOD) δ 8.06 (s, 1H, H₈), 5.95 (s, 1H, H_{1'}), 4.25 (d, *J* = 9.0 Hz, 1H, H_{3'}), 4.05-4.02 (m, 2H, H_{4'} and H_{5'a}), 3.87-3.83 (m, 1H, H_{5'b}), 3.69 (s, 3H, 6NCH₃), 0.96 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 161.83(C6), 160.97 (C2), 152.71 (C4), 139.41 (C8), 119.42 (C5), 93.27 (C1'), 84.17 (C4'), 80.31 (C2'), 73.59 (C3'), 62.27 (C5'), 27.78 (6NCH₃), 20.37 (2'CCH₃).

HPLC (System 1) *t*_R = 7.99 min.

HRMS calculated for C₁₂H₁₈N₆O₄ Na: 333.1378, found 333.1325

Synthesis of 2-amino-6-*N*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.7).

To a solution of 2-amino-6-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine (**5.2**, 2.00 g, 3.19 mmol) in dry DMF (30 ml) was aqueous solution of sodium methanethiolate (1.24 g in 6 ml of H₂O, 19.15 mmol). The reaction mixture was stirred at ambient temperature for 2 h, after that time mixture was diluted with water (50 ml) and extracted 3x with EtOAc (3x 20 ml). Combined organic fractions were dried over anhydrous MgSO₄ and evaporated to dryness under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 94:6) to give the pure compound **5.7** (0.90 g, 86 %) as a white solid.

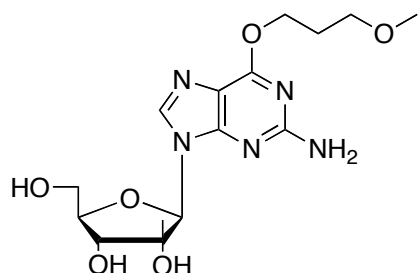
^1H NMR (500 MHz, MeOD) δ 8.32 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.22 (d, J = 9.0 Hz, 1H, H_{3'}), 4.06-4.02 (m, 2H, H_{4'} and H_{5'a}), 3.89-3.88 (m, 1H, H_{5'b}), 2.63 (s, 3H, 6SCH₃), 0.97 (s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, MeOD) δ 164.08 (C6), 163.30 (C2), 151.14 (C4), 140.00 (C8), 125.46 (C5), 92.85 (C1'), 84.20 (C4'), 80.33 (C2'), 73.56 (C3'), 61.15 (C5'), 20.35 (2'CCH₃), 11.47 (6SCH₃).

HPLC (System 1) t_R = 9.96 min.

HRMS calculated for C₁₂H₁₇N₅O₄S Na: 350.0968, found 350.0945.

Synthesis of 2-amino-6-*O*-(3-methoxypropyl)-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.8**).



To a solution of 3-methoxy-1-propanol (ml, 1.17 mmol) in dry THF (10 ml), sodium hydride (1.17 mmol) was added at 0 °C. Reaction was allowed to warm to ambient temperature and was stirred for 30 min, after that time a solution of 2-amino-6-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.3**, 0.12 g, 0.38 mmol) in dry THF (10 ml) was added dropwise. The reaction mixture was stirred at ambient temperature for 8h. After that time reaction was neutralised by the addition of amberlite (H⁺). The mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 85:15) to give the pure compound **5.8** (73 mg, 50 %) as a white solid.

^1H NMR (500 MHz, MeOD) δ 8.27 (s, 1H, H₈), 6.01 (s, 1H, H_{1'}), 4.57 (t, J = 6.5 Hz, 2H, 6OCH₂CH₂CH₂OCH₃), 4.24 (d, J = 9.0 Hz, 1H, H_{3'}), 4.08-4.02 (m, 2H, H_{4'} and H_{5'a}), 3.89-3.86 (m, 1H, H_{5'b}), 3.61 (t, J = 6.5 Hz, 2H, 6OCH₂CH₂CH₂OCH₃), 3.37 (s, 3H, 6OCH₂CH₂CH₂OCH₃), 2.13-2.08 (m, 2H, 6OCH₂CH₂CH₂OCH₃), 0.96 (s, 3H, 2'CCH₃).

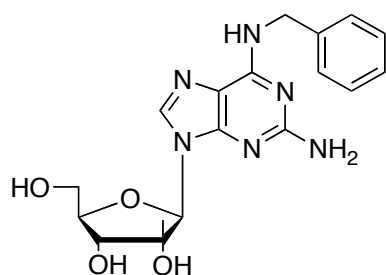
^{13}C NMR (126 MHz, MeOD) δ 162.40 (C6), 161.84 (C2), 154.60 (C4), 139.35 (C8), 115.34 (C5), 93.01 (C1'), 84.15 (C4'), 80.32 (C2'), 73.56 (C3'), 70.23

(6OCH₂CH₂CH₂OCH₃), 61.16 (6OCH₂CH₂CH₂OCH₃), 61.16 (C5'), 58.91 (6OCH₂CH₂CH₂OCH₃), 30.23 (6OCH₂CH₂CH₂OCH₃), 20.35 (2'CCH₃).

HPLC (System 1) *t*_R = 10.42 min.

HRMS Calculated for C₁₅H₂₃N₅O₆Na 392.1574; found: 392.1512.

Synthesis of 2-amino-6-NH-(benzyl)-9-(2'-C-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl) purine (5.9).



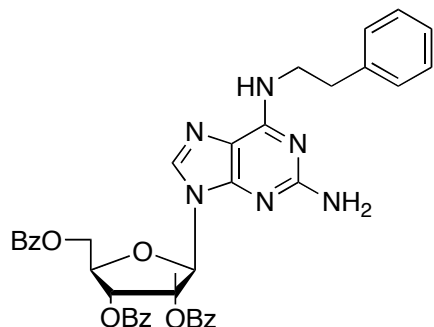
To a suspension of 2-amino-6-chloro-9-(2'-C-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (**5.2**, 1.0 g, 1.60 mmol) in anhydrous ethanol (100 ml), at room temperature, benzylamine (1.75 ml, 10 equiv.) was added. The mixture was stirred under reflux overnight, cooled down to room temperature and ethanol was removed under reduced pressure. The residue was then precipitated from CHCl₃/MeOH to give the pure compound **5.9** (0.57 g, 91 %) as a white solid.

¹H NMR (500 MHz, DMSO) δ 8.03 (s, 1H, H₈), 7.36 (d, *J* = 7.5 Hz, 2H, 6NHCH₂Ph), 7.30 (t, *J* = 7.5 Hz, 2H, 6NHCH₂Ph), 7.21 (t, *J* = 7.5 Hz, 1H, 6NHCH₂Ph), 5.86 (s, 2H, NH₂), 5.80 (s, 1H, H_{1'}), 5.18-5.14 (m, 2H, 3'OH and 5'OH), 5.01 (s, 1H, 2'OH), 4.68-4.62 (m, 2H, 6NHCH₂Ph), 4.02 (d, *J* = 8.1 Hz, 1H, H_{3'}), 3.88-3.85 (m, 2H, H_{4'} and H_{5'a}), 3.75-3.58 (m, 1H, H_{5'b}), 0.81 (s, 3H, CH₃).

HPLC (System 1) *t*_R = 12.61 min.

HRMS calculated for C₁₈H₂₂N₆O₄Na : 409.1600; found 409.1593.

Synthesis of 2-amino-6-NH-(phenylethyl)-9-(2'-C-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (5.10').

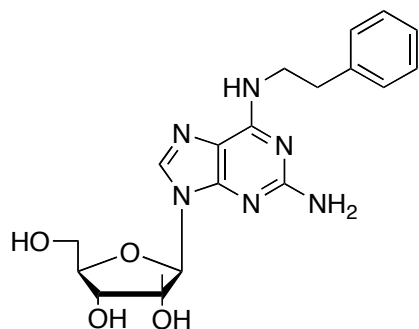


To a suspension of 2-amino-6-chloro-9-(2'-C-methyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)purine (1.00 g, 1.60 mmol) in anhydrous ethanol (100 ml), at room temperature, 2-phenylethylamine was added (2.02 ml, 16.00

mmol). The mixture was stirred under reflux overnight, cooled down to room temperature. Ethanol was removed under reduced pressure. The residue was then precipitated from $\text{CHCl}_3/\text{MeOH}$ to give the pure compound **5.10'** (0.626 g, 55 %) as a white solid.

^1H NMR (500 MHz, MeOD) δ 8.12 (d, $J = 7.2$ Hz, 2H, Bz), 8.03 (d, $J = 7.2$ Hz, 2H, Bz), 7.96 (d, $J = 7.3$ Hz, 2H, Bz), 7.90 (s, 1H, H_8), 7.65 (t, $J = 7.5$ Hz, 1H, Bz), 7.58 – 7.52 (m, 2H, Bz), 7.53 (t, $J = 7.8$ Hz, 2H, Bz), 7.43 (t, $J = 7.8$ Hz, 2H, Bz), 7.39 (t, $J = 7.8$ Hz, 2H, Bz), 7.35–7.26 (m, 5H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$), 6.65 (s, 1H, $\text{H}_{1'}$), 6.28 (d, $J = 6.00$ Hz, 1H, $\text{H}_{3'}$), 5.05–5.02 (m, 1H, $\text{H}_{5'a}$), 4.84 – 4.67 (m, 3H, $\text{H}_{5'b}$, H_4'), 3.95–3.89 (m, 2H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$), 3.01 (t, $J = 7.0$ Hz, 2H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$) 1.65 (s, 3H, $2'\text{CCH}_3$).

Synthesis of 2-amino-6-*NH*-(phenylethyl)-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.10**).**



To a solution of protected nucleoside **5.10'** (0.628 g, 0.88 mmol) in anhydrous methanol (10 ml) at room temperature was added sodium methoxide (0.15 g, 2.82 mmol). After being stirred for 2 hours, the solvent was removed under reduced pressure and the residue chromatographed on silica gel ($\text{CHCl}_3/\text{MeOH}$, 92:8) to yield the pure unprotected nucleoside (0.332 g, 94 %) as a white solid.

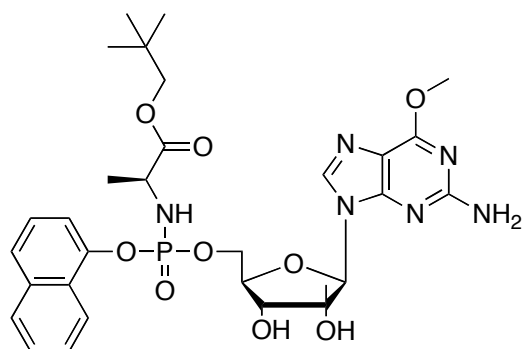
^1H NMR (500 MHz, MeOD) δ 8.20 (s, 1H, H_8), 7.32 – 7.19 (m, 5H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$), 5.96 (s, 1H, $\text{H}_{1'}$), 4.29 (d, $J = 8.0$ Hz, 1H, $\text{H}_{3'}$), 4.13–4.05 (m, 3H, H_4' and $\text{H}_{5'a}$), 3.95 – 3.85 (m, 2H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$), 3.04 (t, $J = 7.0$ Hz, 2H, $6\text{NHCH}_2\text{CH}_2\text{Ph}$) 0.97 (s, 3H, $2'\text{CCH}_3$).

HPLC (System 1) $t_R = 13.55$ min.

HRMS calculated for $\text{C}_{19}\text{H}_{24}\text{N}_6\text{O}_4\text{Na}$: 423.1757; found 423.1763

CHN calculated for $\text{C}_{19}\text{H}_{24}\text{N}_6\text{O}_4 + 3.5 \text{ H}_2\text{O}$: C 49.24, H 6.74, N 18.13; found: C 49.13, H 6.83, N 18.05.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (2.15**, INX-189).**



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 584 mg, 1.88 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 2.16 g, 5.63

mmol), NMI (0.74 ml, 9.38 mmol) in 15 ml of dry THF. Crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient). To give a pure product **2.15** as white foam (139 mg, 11%).

³¹P NMR (202 MHz, MeOD) δ 4.33, 4.29.

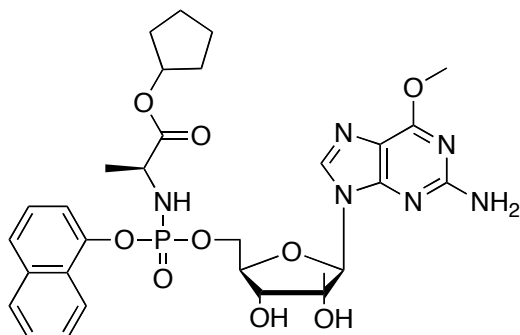
¹H NMR (500 MHz, MeOD) δ 8.19-8.15 (m, 1H, H₈-naph), 7.98, 7.96 (2s, 1H, H₈), 7.86-7.82 (m, 1H, H₅-Naph), 7.68, 7.65 (2d, J = 7.0 Hz, 1H, H₄-Naph), 7.53-7.44 (m, 3H, H₂, H₇, H₆-Naph), 7.39, 7.37 (2t, J = 8.0 Hz, 1H, H₃-Naph), 6.01, 6.00 (2s, 1H, H₁'), 4.67-4.64 (m, 2H, H_{5'}), 4.63-4.59 (m, 2H, H_{3'}, H_{4'}), 4.09-4.05 (m, 1H, CHCH₃), 4.04 (s, 3H, 6OCH₃), 3.75, 3.72, 3.64, 3.58 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.33 (d, J = 7.5 Hz, 3H, CHCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃), 0.85, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.06, 174.80 (2d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.73 (C6), 161.86 (C2), 154.55, 154.51 (C4), 148.00, 147.95 (d, $^2J_{C-O-P}$ = 3.8 Hz, ipso Naph), 139.36, 139.08 (C8), 136.27, 136.25 (C10-Naph), 128.86, 128.80 (CH-Naph), 127.88, 127.73 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.48, 126.53, 126.49, 125.97, 122.81, 122.76 (CH-Naph), 116.24, 116.22 (C2-Naph), 116.19, 115.63 (C5), 93.34, 93.18 (C1'), 82.32, 82.16 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.99, 79.95 (C2'), 75.52, 75.37 (OCH₂C(CH₃)₃), 74.95, 74.70 (C3'), 68.11, 67.62 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 54.28, 54.07 (6OCH₃), 51.79, 51.71 (CHCH₃), 32.26, 32.22 (OCH₂C(CH₃)₃), 26.74, 26.71 (OCH₂C(CH₃)₃), 20.89, 20.69 (2d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.39, 20.35 (2'CCH₃).

HPLC (System 1) t_R = 20.95, 21.48 min

HRMS calculated for C₃₀H₃₉N₆O₉PNa: 681.2457; found 681.2472.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(cyclopentoxo-L-alaninyl)] phosphate (5.12a).



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(**5.4**, 2'-*C*-methyl- β -D-ribofuranosyl) purine (250 mg, 0.80 mmol), α -naphthyl-(cyclopentoxo-L-alaninyl) phosphorochloridate (**3.3h**, 920 mg, 2.40

mmol), NMI (0.47 ml, 4.02 mmol) in 5 ml of dry THF. Crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product **5.12a**, as white foam (75 mg, 14%).

³¹P NMR (202 MHz, MeOD) δ 4.36, 4.20.

¹H NMR (500 MHz, MeOD) δ 8.19- 8.15 (m, 1H, H₈-naph), 7.98, 7.96 (2s, 1H, H₈), 7.86–7.82 (m, 1H, H₅-Naph), 7.68, 7.65 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.53–7.44 (m, 3H, H₂, H₇, H₆-Naph), 7.39, 7.37 (2t, J = 8.0 Hz, 1H, H₃-Naph), 6.02, 6.00 (2s, 1H, H₁'), 5.00-4.97 (m, 1H, OCH ester), 4.66- 4.58 (m, 2H, H₅'), 4.37- 4.24 (m, 2H, H₃', H₄'), 4.04 (s, 3H, 6OCH₃), 4.01- 3.93 (m, 1H, CHCH₃), 1.78- 1.69 (m, 2H, CH₂ ester), 1.60- 1.47 (m, 6H, 3x CH₂ ester), 1.28 (d, J = 7.0 Hz, 3H, CHCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃).

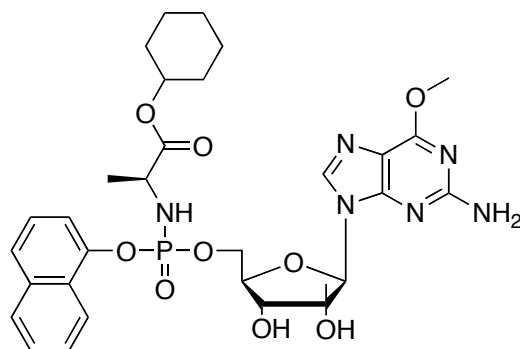
¹³C NMR (126 MHz, MeOD) δ 174.87, 174.62 (2d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.73 (C6), 161.86 (C2), 154.55, 154.50 (C4), 148.01, 147.95 (d, $^2J_{C-O-P}$ = 3.8 Hz, ipso Naph), 139.38, 139.08 (C8), 136.27, 136.24 (C10-Naph), 128.86, 128.81 (CH-Naph), 127.86, 127.78 (2d, $^3J_{C-C-O-P}$ = 5.0 Hz, C9-Naph), 127.73, 127.47, 126.52, 126.48, 125.96, 125.93, 122.78, 122.74 (CH-Naph), 116.21, 116.13 (2d, $^3J_{C-C-O-P}$ = 3.8 Hz, C2-Naph), 115.63, 115.60 (C5), 93.37, 93.19 (C1'), 82.31, 82.16 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 80.00, 79.96 (C2'), 79.52, 79.50 (OCH ester), 74.95, 74.71 (C3'), 68.06, 67.59 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 54.30, 54.29 (6OCH₃), 51.81, 51.75 (CHCH₃) 33.52, 33.39 (CH₂ ester), 24.60, 24.58 (CH₂ ester), 20.67, 20.63 (2d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.44, 20.36 (2'CCH₃).

HPLC (System 1) t_R = 19.52, 19.96 min.

MS (ES+) m/z : 657.24 (MH^+ , 100%)

HRMS calculated for $C_{30}H_{39}N_6O_9PNa$: 681.2457; found 681.2472.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (5.12d**).**



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (980mg, 3.15 mmol), α -naphthyl-(cyclohexoxy-L-alaninyl) phosphorochloridate (**3.3i**, 3.82 g, 9.44

mmol), NMI (1.25 ml, 15.74 mmol) in 5 ml of dry THF. Crude mixture was purified by column chromatography, using $CHCl_3/MeOH$ (0 to 5%, gradient), to give a pure product **5.12b** as white foam (730 mg, 14%).

^{31}P NMR (202 MHz, MeOD) δ 4.34, 4.26.

1H NMR (500 MHz, MeOD) δ 8.19-8.15 (m, 1H, H_8 -naph), 7.98, 7.96 (2s, 1H, H_8), 7.84-7.82 (m, 1H, H_5 -Naph), 7.67, 7.65 (2d, $J = 8.0$ Hz, 1H, H_4 -Naph), 7.53-7.44 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.41-7.36 (m, 1H, H_3 -Naph), 6.02, 6.00 (2s, 1H, $H_{1'}$), 4.69-4.60 (m, 3H, CH ester and $H_{5'}$), 4.35- 4.29 (m, 2H, $H_{3'}$, $H_{4'}$), 4.04 (s, 3H, $6OCH_3$), 4.00 (q, $J = 7.8$ Hz, 1H, $CHCH_3$), 1.69- 1.60 (m, 4H, 2x CH_2 ester), 1.31- 1.19 (m, 9H, 3x CH_2 ester and $CHCH_3$), 0.98, 0.96 (2s, 3H, $2'CCH_3$).

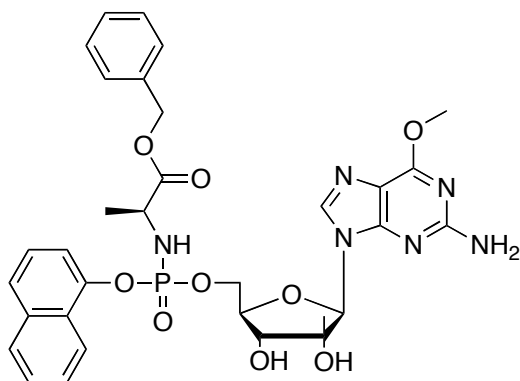
^{13}C NMR (126 MHz, MeOD) δ 174.51, 174.25 (2d, $^3J_{C-C-N-P} = 5.0$ Hz, C=O), 162.72 (C6), 161.86 (C2), 154.55, 154.50 (C4), 148.03, 148.01 (d, $^2J_{C-O-P} = 3.8$ Hz, ipso Naph), 139.33, 139.05 (C8), 136.28, 136.25 (C10-Naph), 128.85, 128.79 (CH-Naph), 127.88, 127.73 (2d, $^3J_{C-C-O-P} = 6.3$ Hz, C9-Naph), 127.46, 126.82, 126.53, 126.48, 125.95, 125.91, 122.81, 122.76, 122.27 (CH-Naph), 116.21, 116.12 (2d, $^3J_{C-C-O-P} = 3.8$ Hz, C2-Naph), 115.62, 115.59 (C5), 93.35, 93.17 (C1'), 82.31, 82.15 (2d, $^3J_{C-C-O-P} = 8.8$ Hz, C4'), 79.99, 79.96 (C2'), 74.94, 74.92 (CH ester), 74.95, 74.71 (C3'), 68.05, 67.56 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 54.29 ($6OCH_3$), 51.85, 51.79 (C α Ala), 32.35, 32.39 (CH_2 ester), 26.35 (CH_2 ester), 24.57 (CH_2 ester), 20.81, 20.60 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, $CHCH_3$), 20.36, 20.33 ($2'CCH_3$).

HPLC (System 1) t_R = 21.28, 21.83 min.

MS (ES+) m/z : 671.26 (MH^+ , 100%).

HRMS calculated for $C_{31}H_{40}N_6O_9P_1$: 671.2594, found 671.2584.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy -L-alaninyl)] phosphate (4.4e).



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 150 mg, 0.482 mmol), α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3k**, 584 mg, 1.44 mmol), NMI (0.19 ml, 2.41 mmol) in 5 ml of dry THF. The crude

mixture was purified by column chromatography, using $CHCl_3$ /MeOH (0 to 5%, gradient), to give a pure product as white foam (**5.12c**, 45 mg, 14%).

^{31}P NMR (202 MHz, MeOD) δ 4.27, 4.26.

1H NMR (500 MHz, MeOD) δ 8.17, 8.15 (2d, J = 7.5 Hz, 1H, H_8 -Naph), 7.97, 7.93 (2s, 1H, H_8), 7.89–7.85 (m, 1H, H_5 -Naph), 7.69, 7.67 (2d, J = 8.5 Hz, 1H, H_4 -Naph), 7.54–7.44 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.37, 7.36 (2t, J = 8.0 Hz, 1H, H_3 -Naph), 7.28–7.23 (m, 5H, OCH_2Ph), 5.99, 5.98 (2s, 1H, $H_{1'}$), 5.06–4.95 (m, 2H, OCH_2Ph), 4.61–4.56 (m, 2H, $H_{5'}$), 4.34, 4.27 (2d, J = 9.0 Hz, 1H, $H_{3'}$), 4.25–4.21 (m, 1H, $H_{4'}$), 4.10–4.06 (m, 1H, $CHCH_3$), 4.04, 4.03 (2s, 3H, $6OCH_3$), 1.30, 1.29 (2d, J = 7.0 Hz, $CHCH_3$), 0.98, 0.95 (2s, 3H, $2'CCH_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.85 (d, $^3J_{C-C-N-P}$ = 4.4, C=O), 174.62 (d, $^3J_{C-C-N-P}$ = 5.5, C=O), 162.74 (C6), 161.83 (C2), 154.56, 154.51 (C4), 147.99, 147.86 (2d, $^2J_{C-O-P}$ = 1.9 Hz, ipso Naph), 139.31, 139.04 (C8), 137.08 (ipso OCH_2Ph), 136.24, 136.22 (C10-Naph), 129.54, 129.26, 129.25, 129.17, 129.12, 128.87, 128.83 (CH-Naph and OCH_2Ph), 127.90, 17.85 (2d, $^3J_{C-C-O-P}$ = 4.1, C9-Naph), 127.77 (CH-Naph), 127.53, 126.56, 126.53, 126.00, 122.83, 122.76 (CH-Naph and OCH_2Ph), 116.28, 116.27 (C2-Naph), 115.68, 115.65 (C5), 93.30, 93.12 (C1'), 82.29, 82.12 (2d, $^3J_{C-C-O-P}$ = 8.0

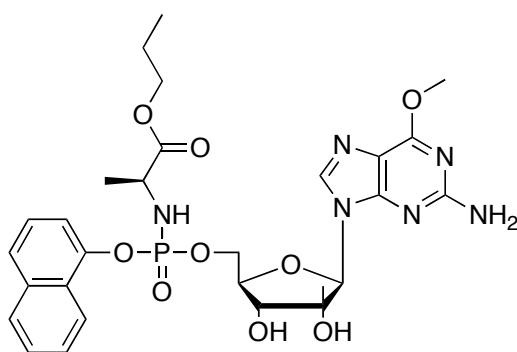
Hz, C4'), 80.03, 79.99 (C2'), 79.53 (6OCH₃), 74.96, 74.64 (C3'), 68.03 (d, $^2J_{C-O-P}$ = 4.7 Hz, C5'), 67.98, 67.96 (OCH₂Ph), 67.48, 67.12 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 51.84, 51.75 (CHCH₃), 20.87 (d, $^3J_{C-C-N-P}$ = 6.1 Hz, CHCH₃), 20.68 (d, $^3J_{C-C-N-P}$ = 4.6, CHCH₃), 20.36, 20.33 (2'CCH₃).

HPLC (System 1) t_R = 18.51, 18.92 min.

MS (ES+) m/z : 701.21 (MNa⁺, 100%).

HRMS calculated for C₃₂H₃₅N₆O₉P₁Na: 701.2101, found 701.2119.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(propoxy-L-alaninyl)] phosphate (5.12d).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 250 mg, 0.803 mmol), α-naphthyl-(propoxy-L-alaninyl) phosphorochloridate (**3.3c**, 571 mg, 1.60 mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF.

The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product **5.12d** as white foam (163 mg, 32%).

³¹P NMR (202 MHz, MeOD) δ 4.33, 4.22.

¹H NMR (500 MHz, MeOD) δ 8.19–8.15 (m, 1H, H₈-naph), 7.99, 7.97 (2s, 1H, H₈), 7.90–7.82 (m, 1H, H₅-Naph), 7.68, 7.65 (2d, J = 8.5 Hz, 1H, H₄-Naph), 7.54–7.45 (m, 3H, H₂, H₇, H₆-Naph), 7.38, 7.36 (2t, J = 8.0 Hz, 1H, H₃-Naph), 6.04, 6.03 (2s, 1H, H₁'), 4.70–4.61 (m, 2H, H₅'), 4.37–4.26 (m, 2H, H₃', H₄'), 4.10–4.04 (m, 1H, CHCH₃), 4.03 (s, 3H, 6OCH₃), 3.95–3.84 (m, 2H, OCH₂CH₂CH₃), 1.53–1.45 (m, 2H, OCH₂CH₂CH₃), 1.31 (d, J = 7.0 Hz, 3H, CHCH₃), 0.99, 0.97 (2s, 3H, 2'CCH₃), 0.82 (t, J = 7.5 Hz, 3H, OCH₂CH₂CH₃).

¹³C NMR (126 MHz, MeOD) δ 175.13, 174.89 (2d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.75 (C6), 161.84 (C2), 156.56, 154.52 (C4), 148.02, 147.96 (2d, $^2J_{C-O-P}$ = 3.0 Hz, ipso Naph), 139.33, 139.07 (C8), 136.25, 136.22 (C10-Naph), 128.86, 128.82, (CH-Naph), 127.85, 127.76 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.49, 126.55, 126.51, 125.97,

125.95, 122.81, 122.76 (CH-Naph), 116.24, 116.17 (2d, $^3J_{C-C-O-P} = 4.5$ Hz, C2-Naph), 115.67, 115.66 (C5), 93.32, 93.17 (C1'), 82.30, 82.16 (2d, $^3J_{C-C-O-P} = 8.8$ Hz, C4'), 80.04, 80.01 (C2'), 74.94, 74.71 (C3'), 68.00 (OCH₂CH₂CH₃), 67.87, 67.54 (d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 54.36 (6OCH₃), 51.77, 51.17 (CHCH₃), 20.84, 20.62 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃), 20.46, 20.44 (2'CCH₃), 10.67, 10.65 (OCH₂CH₂CH₃).

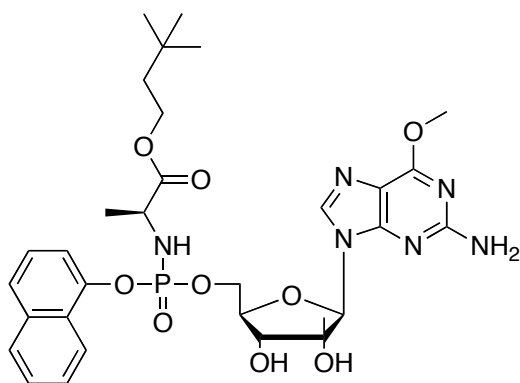
HPLC (System 1) $t_R = 18.51, 18.95$ min.

HPLC (System 2) $t_R = 24.63, 25.49$ min.

MS (ES+) m/z : 653.20 (MNa⁺, 100%).

HRMS calculated for C₃₀H₃₆N₆O₉P₁Na: 653.2101, found 653.2073.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl)] phosphate (5.12e).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 250 mg, 0.803 mmol), α-naphthyl-(3,3-dimethyl-1-butoxy-L-alaninyl) phosphorochloridate (**3.3f**, 640 mg, 1.60 mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in

5 ml of dry THF. The crude mixture was purified by column chromatography using CHCl₃/MeOH (95:5), to give a pure product **5.12e**, as white foam (190 mg, 35%).

³¹P NMR (202 MHz, MeOD) δ 4.33, 4.29.

¹H NMR (500 MHz, MeOD) δ 8.20- 8.16 (m, 1H, H₈-naph), 8.00, 7.98 (2s, 1H, H₈), 7.85- 7.81 (m, 1H, H₅-Naph), 7.66, 7.64 (2d, $J = 8.5$ Hz, 1H, H₄-Naph), 7.54- 7.45 (m, 3H, H₂, H₇, H₆-Naph), 7.39, 7.37 (2t, $J = 8.0$ Hz, 1H, H₃-Naph), 6.04, 6.03 (2s, 1H, H₁'), 4.71- 4.61 (m, 1H, H₅'), 4.36- 4.27 (m, 1H, H₃', H₄'), 4.03 (s, 3H, 6OCH₃), 4.02 - 3.91 (m, 2H, CHCH₃ and OCH₂CH₂C(CH₃)₃), 1.39-1.29 (m, 5H, CHCH₃ and OCH₂CH₂C(CH₃)₃), 0.99, 0.97 (2s, 3H, 2'CCH₃), 0.82, 0.81 (2s, 9H, OCH₂CH₂C(CH₃)₃).

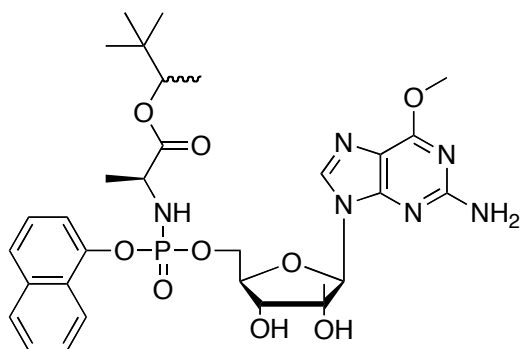
^{13}C NMR (126 MHz, MeOD) δ 175.06, 174.80 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, C=O), 162.73 (C6), 161.86 (C2), 154.55, 154.51 (C4), 148.00, 147.95 (d, $^2J_{\text{C-O-P}} = 3.8$ Hz, ipso Naph), 139.36, 139.08 (C8), 136.27, 136.25 (C10-Naph), 128.86, 128.80 (CH-Naph), 127.88, 127.73 (2d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.78, 127.76, 127.51, 126.51, 125.53, 125.98, 125.93, 122.83, 122.79 (CH-Naph), 116.22, 116.09 (2d, $^3J_{\text{C-C-O-P}} = 3.8$ Hz, C2-Naph), 115.67 (C5), 93.30, 93.16 (C1'), 82.29, 82.15 (2d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C4'), 80.06, 80.03 (C2'), 74.90, 74.67 (C3'), 67.97, 67.50 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 64.01, 63.99 (OCH₂CH₂C(CH₃)₃), 54.37, 54.35 (6OCH₃), 51.74, 51.71 (CHCH₃), 42.67, 42.60 (OCH₂CH₂C(CH₃)₃), 30.08, 30.00 (OCH₂CH₂C(CH₃)₃), 20.73, 20.52 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH₃), 20.46, 20.44 (2'CCH₃).

HPLC (System 1) $t_{\text{R}} = 22.03, 22.49$ min

MS (ES+) m/z : 695.27 (MNa⁺, 100%).

HRMS calculated for C₃₁H₄₂N₆O₉P₁: 673.2751, found 673.2752.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(3,3-dimethyl-2-butoxy-L-alaninyl)] phosphate (5.12f**).**



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(3,3-dimethyl-2-butoxy-L-alaninyl) phosphorochloridate (**3.3g**, 960mg, 1.60 mmol), *t*BuMgCl (1.61 ml,

1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (95:5), to give a pure product **5.12f** as a white foam (195 mg, 36%).

^{31}P NMR (202 MHz, CDCl₃) δ 4.33, 4.29.

^1H NMR (500 MHz, MeOD) δ 8.19- 8.15 (m, 1H, H₈-naph), 7.99, 7.98, 7.96 (3s, 1H, H₈), 7.86- 7.80 (m, 1H, H₅-Naph), 7.67- 7.63 (m, 1H, H₄-Naph), 7.53 – 7.46 (m, 3H, H₂, H₇, H₆-Naph), 7.39- 7.34 (m, 1H, H₃-Naph), 6.01, 6.00 (2s, 1H, H₁'), 4.69- 4.57 (m, 3H, H₅', and OCH(CH₃)C(CH₃)₃), 4.36- 4.28 (m, 2H, H₃', H₄'), 4.05 – 4.01 (m,

^1H , $\text{H}\alpha$), 4.03 (s, 3H, 6OCH_3), 1.33, 1.31 (2d, $J = 7.0$ Hz, 3H, CHCH_3), 1.05–0.98 (m, 3H, $\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$) 0.95, 0.94, 0.91 (3s, 3H, $2'\text{CCH}_3$), 0.84, 0.83, 0.80, 0.79 (4s, 9H, $\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$).

^{13}C NMR (126 MHz, MeOD) δ 175.06, 174.80 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, C=O), 162.73 (C6), 161.86 (C2), 154.55, 154.51 (C4), 148.00, 147.95 (d, $^2J_{\text{C-O-P}} = 3.8$ Hz, ipso Naph), 139.36, 139.08 (C8), 136.27, 136.25 (C10-Naph), 128.86, 128.80 (CH-Naph), 127.88, 127.73 (2d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.48, 126.55, 126.49, 125.98, 122.84, 122.79 (CH-Naph), 116.28, 116.23 (C2-Naph), 116.20, 115.68 (C5), 93.35, 93.19 (C1'), 82.32, 82.17 (2d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 80.16, 80.07 ($\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 80.01, 79.98 (C2'), 75.52, 75.37 ($\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 75.00, 74.75 (C3'), 68.09 (d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 67.66 (C5'), 54.28 (6OCH_3), 51.94, 51.87 (d, $^2J_{\text{C-N-P}} = 7.5$ Hz, CHCH_3), 35.16, 35.14, 35.07, 35.03 ($\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$), 21.05, 20.69 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH_3), 20.39, 20.35 ($2'\text{CCH}_3$), 15.27, 15.19, 15.13, 15.07 ($\text{OCH}(\text{CH}_3)\text{C}(\text{CH}_3)_3$).

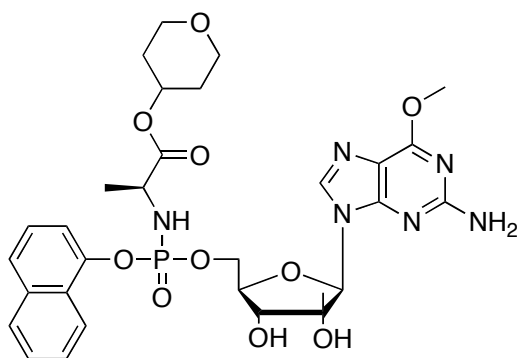
HPLC (System 1) $t_R = 21.73$ min.

HPLC (System 2) $t_R = 28.21, 28.91$ min.

MS (ES+) m/z : 695.26 (MNa^+ , 100%).

HRMS calculated for $\text{C}_{31}\text{H}_{42}\text{N}_6\text{O}_9\text{P}_1\text{Na}$: 695.2570, found 695.2551.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(tetrahydropyroxyl-L-alaninyl)] phosphate (5.12g).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(tetrahydropyroxyl-L-alaninyl) phosphorochloridate (**3.3j**, 640 mg, 1.60 mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in

5 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (95:5), to give a pure product **5.12g** as a white foam (142 mg, 26%).

^{31}P NMR (202 MHz, MeOD) δ 4.25, 4.12.

^1H NMR (500 MHz, MeOD) δ 8.19- 8.16 (m, 1H, H₈-naph), 7.99, 7.96 (2s, 1H, H₈), 7.87– 7.82 (m, 1H, H₅-Naph), 7.68, 7.66 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.58 – 7.45 (m, 3H, H₂, H₇, H₆-Naph), 7.40, 7.38 (2t, J = 8.0 Hz, 1H, H₃-Naph), 6.02, 6.00 (2s, 1H, H_{1'}), 4.81- 4.73 (m, 1H, OCH ester), 4.69- 4.58 (m, 2H, H_{5'}), 4.36- 4.24 (m, 2H, H_{3'}, H_{4'}), 4.07- 3.99 (m, 1H, CHCH₃), 4.05, 4.04 (2s, 3H, 6OCH₃), 3.80- 3.73 (m, 2H, CH₂ ester), 3.47- 3.39 (m, 2H, CH₂ ester), 1.79- 1.70 (m, 2H, CH₂ ester), 1.54- 1.44 (m, 2H, CH₂ ester), 1.32, 1.30 (2d, J = 6.5 Hz, 3H, CHCH₃), 0.98, 0.97 (2s, 2'CCH₃).

^{13}C NMR (126 MHz, MeOD) δ 174.31, 174.07 (2d, $^3J_{\text{C-C-N-P}}$ = 5.0 Hz, C=O), 162.73 (C6), 161.89, 161.87 (C2), 154.58, 154.53 (C4), 148.00, 147.96 (ipso Naph), 139.39, 139.11 (C8), 136.28, 136.25 (C10-Naph), 128.85, 128.79 (CH-Naph), 127.86, 127.77 (2d, $^3J_{\text{C-C-O-P}}$ = 5.0 Hz, C9-Naph), 127.49, 127.25, 126.97, 126.69, 126.54, 126.51, 125.98, 125.92, 122.79, 122.74 (CH-Naph), 116.24, 116.12 (2d, $^3J_{\text{C-C-O-P}}$ = 3.8 Hz, C2-Naph), 115.61 (C5), 93.37, 93.20 (C1'), 82.34, 82.16 (2d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C4'), 79.99, 79.95 (C2'), 74.96, 74.73 (OCH ester), 71.33 (C3'), 68.14, 67.65 (2d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C5'), 66.04, 66.02 (CH₂O ester), 54.29 (6OCH₃), 51.85, 51.76 (CHCH₃), 32.52, 32.48 (CH₂ ester), 20.67, 20.50 (2d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, CHCH₃), 20.35, 20.31 (2'CCH₃).

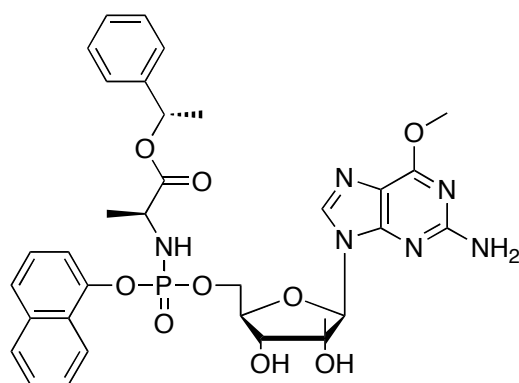
HPLC (System 1) t_{R} = 16.72, 17.08 min.

HPLC (System 2) t_{R} = 22.55, 23.49 min.

MS (ES+) m/z : 695.22 (MNa⁺, 100%).

HRMS calculated for C₃₀H₃₇N₆O₁₀P₁Na: 695.2207, found 695.2217.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(*S*-phenylethoxy-L-alaninyl)] phosphate (5.12h).



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.4, 1.50 g, 4.82 mmol), α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (3.2I, 6.04 g, 14.46 mmol), *t*BuMgCl (9.64 ml, 9.64 mmol) in 50 ml of dry THF. The

crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product **5.12h** as a white foam (571mg, 17%).

³¹P NMR (202 MHz, MeOD) δ 4.30, 4.27.

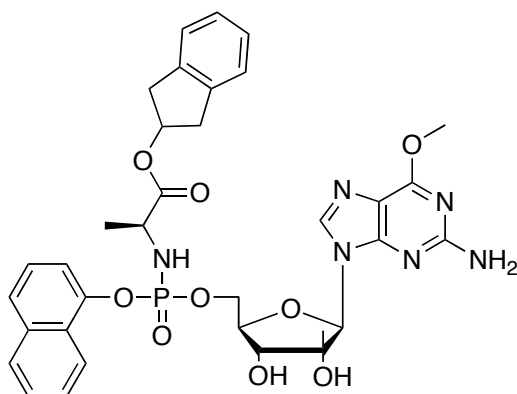
¹H NMR (500 MHz, MeOD) δ 8.20- 8.16 (m, 1H, H₈-Naph), 7.98, 7.94 (2s, 1H, H₈), 7.92- 7.85 (m, 1H, H₅-Naph), 7.71, 7.68 (2d, *J* = 8.0 Hz, 1H), 7.54 – 7.46 (m, 3H, H₂, H₇, H₆-Naph), 7.40, 7.39 (2t, *J* = 8.0 Hz, 1H, H₃-Naph), 7.30- 7.22 (m, 5H, OCH(CH₃)Ph), 6.00, 5.99 (2s, 1H, H_{1'}), 5.74, 5.68 (2q, *J* = 7.0 Hz, 1H, OCH(CH₃)Ph), 4.62– 4.55 (m, 1H, H_{5'}), 4.33, 4.29 (2d, *J* = 9.0 Hz, 1H, H_{3'}), 4.26- 4.21 (m, 1H, H_{4'}), 4.07- 4.01 (m, 1H, CHCH₃), 4.05, 4.04 (2s, 3H, 6OCH₃), 1.40, 1.41 (2d, *J* = 6.0 Hz, 3H, OCH(CH₃)Ph), 1.26, 1.24 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃).

HPLC (System 1) t_R = 20.87, 21.39 min.

MS (ES+) *m/z*: 693.25 (MH⁺, 100%).

HRMS calculated for C₃₃H₃₈N₆O₉P₁: 693.2438, found 693.2468.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(2-indanoxy-*L*-alaninyl)] phosphate (5.12i**).**



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α-naphthyl-(2-indanoxy-*L*-alaninyl) phosphorochloridate (**3.3m**, 690 mg, 1.60 mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF.

The crude mixture was purified by column chromatography, using CHCl₃/MeOH (95:5), to give a pure product **5.12i** as white foam (255.4 mg, 45%).

³¹P NMR (202 MHz, MeOD) δ 4.26, 4.25.

¹H NMR (500 MHz, MeOD) δ 8.17 – 8.10 (m, 1H, H₈-Naph), 7.95, 7.93 (2s, 1H, H₈), 7.81 (d, *J* = 8.5 Hz, 1H, H₅-Naph), 7.65, 7.63 (2d, *J* = 8.5 Hz, 1H, H₄-Naph), 7.50 –

7.42 (m, 3H, H₇, H₆, H₂-Naph), 7.37- 7.33 (m, 1H, H₃-Naph), 7.15- 7.08 (m, 4H, Ar ester), 6.00 (s, 1H, H_{1'}), 5.34- 5.31 (m, 1H, OCH ester), 4.59 – 4.51 (m, 2H, H_{5'}), 4.33 – 4.21 (m, 2H, H_{3'} and H_{4'}), 4.02, 4.01 (2s, 3H, 6OCH₃), 3.98- 3.95 (m, 1H, CHCH₃), 3.20-3.01 (m, 2H, CH₂ ester), 2.85- 2.78 (m, 2H, CH₂ ester), 1.26, 1.25 (2d, *J* = 7.00 Hz, 3H, CHCH₃), 0.97, 0.94 (2s, 3H, 2'-CH₃).

¹³C NMR (126 MHz, MeOD) δ 174.97, 174.73 (d, ³*J*_{C-C-N-P} = 3.8, C=O), 162.73 (C6), 161.83 (C2), 154.53, 154.48 (C4), 147.97, 147.91 (d, ²*J*_{C-O-P} = 3.8 Hz, ipso Naph), 141.52, 141.48, 141.36, 141.35, 139.28, 138.97, 136.24, 136.22, 128.87, 128.83, 127.88, 127.82, 127.81, 127.77, 127.52, 126.55, 126.51, 125.99, 125.60, 125.57, 125.52, 122.81, 122.75 (Naph, C9-Naph, 2 ipso Ar ester and 4x CH Ar), 116.24, 16.22 (d, ³*J*_{C-C-O-P} = 3.8 Hz, C2-Naph), 115.67, 115.65 (C5), 93.32, 93.11 (C1'), 82.28, 82.08 (2d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 79.98, 79.96 (C2'), 77.73, 77.69 (OCH ester), 74.91, 74.57 (C3'), 67.97, 67.34 (2d, ²*J*_{C-O-P} = 5.0, C5'), 54.36, 54.34 (6OCH₃), 51.82, 51.71 (CHCH₃), 40.49, 40.44, 40.33, 40.25 (CH₂ ester), 20.63, 20.49 (CHCH₃), 20.4, 20.3 (2'CCH₃),

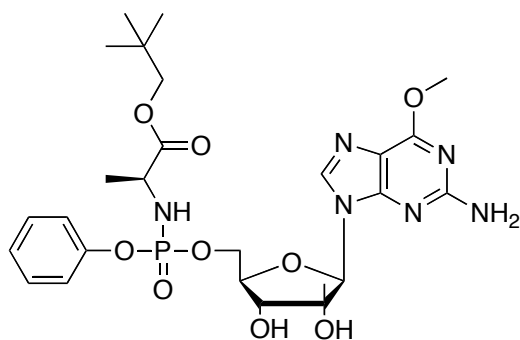
HPLC (System 1) *t*_R = 20.81, 21.28 min.

HPLC (System 2) *t*_R = 27.75, 28.16 min

MS (ES+) *m/z*: 727.23 (MNa⁺, 100%).

HRMS calculated for C₃₄H₃₈N₆O₉P₁: 705.2438, found 705.2468.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[phenyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.12j).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), phenyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3n**, 650 mg, 1.60

mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (95:5), to give a pure product **5.12j** as white foam (97.2 mg, 20%).

^{31}P NMR (202 MHz, MeOD) δ 4.04, 3.89.

^1H NMR (500 MHz, MeOD) δ 7.96, 7.95 (2s, 1H, H_8), 7.36–7.32 (m, 2H, *Ph*), 7.28 – 7.26 (m, 2H, *Ph*), 7.19 – 7.15 (m, 1H, *Ph*), 6.03, 6.00 (2s, 1H, $\text{H}_{1'}$), 4.63– 4.50 (m, 1H, H_5'), 4.30– 4.20 (m, 1H, H_3' , H_4'), 4.07, 4.06 (2s, 3H, 6OCH_3), 4.03 – 4.06 (m, 1H, CHCH_3), 3.83, 3.80, 3.72, 3.66 (2AB, $J_{\text{AB}} = 10.50$ Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.36, 1.35 (2d, $J = 7.50$ Hz, 3H, CHCH_3), 0.99, 0.96 (2s, 3H, $2'\text{CCH}_3$), 0.91, 0.88 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

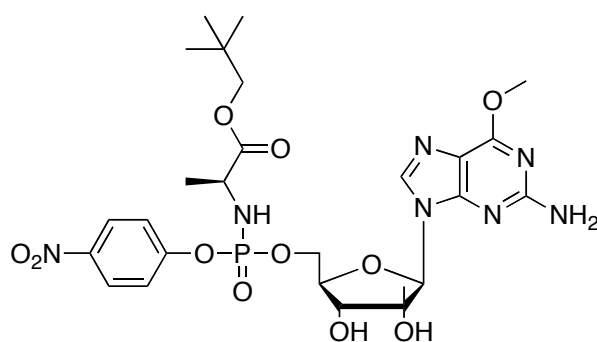
^{13}C NMR (126 MHz, MeOD) δ 175.07, 174.85 (2d, $^3J_{\text{C-C-N-P}} = 5.00$ Hz, C=O), 162.75 (C_6), 161.93, 161.89 (C_2), 154.63 (C_4), 152.21, 152.15 (d, $^2J_{\text{C-O-P}} = 3.80$ Hz, ipso *Ph*), 139.26, 138.92 (C_8), 130.28, 130.78, 126.15, 121.50, 121.47 (*Ph*), 115.59, 115.47 (C_5), 93.24, 92.91 ($\text{C}_{1'}$), 82.23, 82.03 (2d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C_4'), 80.04, 19.97 (C_2'), 75.42, 75.39 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 74.77, 74.28 (C_3'), 67.71, 66.80 (2d, $^2J_{\text{C-O-P}} = 5.00$ Hz, C_5'), 54.29 (6OCH_3), 51.73, 51.60 (CHCH_3), 32.32, 32.25 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 26.77, 26.73 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 20.86, 20.65 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH_3), 20.33 ($2'\text{CCH}_3$).

HPLC (System 1) $t_{\text{R}} = 19.71, 20.16$ min.

MS (ES+) m/z : 609.23 (MH^+ , 100%).

HRMS calculated for $\text{C}_{26}\text{H}_{38}\text{N}_6\text{O}_9\text{P}_1$: 609.2438; found 609.2443.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[*p*-NO₂-phenol-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.12k).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 500 mg, 1.61 mmol), *p*-NO₂-phenyl-(2,2-dimethylpropoxy-L-alaninyl)

phosphorochloridate (**3.3o**, 1.22 g, 3.21 mmol), *t*BuMgCl (3.22 ml, 3.22 mmol) in 10 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (95:5), to give a pure product **5.12k** as a white foam (370 mg, 35%).

^{31}P NMR (202 MHz, MeOD) δ 3.58, 3.51.

^1H NMR (500 MHz, MeOD) δ 8.21 (2d, J = 9.2 Hz, 2H, H_3 and $\text{H}_5\text{-Ph}$) 7.93, 7.92 (2s, 1H, H_8), 7.46, 7.45 (2d, J = 9.2 Hz, 2H, H_2 and $\text{H}_6\text{-Ph}$), 5.99, 5.96 (2s, 1H, $\text{H}_{1'}$), 4.62 - 4.56 (m, 2H, $\text{H}_{5'}$), 4.36- 4.21 (m, 2H, $\text{H}_{3'}$, $\text{H}_{4'}$), 4.07, 4.06 (2s, 3H, 6OCH_3), 4.05- 4.01 (m, 1H, CHCH_3), 3.83, 3.79, 3.74, 3.65 (2AB, J = 10.5 Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.39 (d, J = 7.5 Hz, CHCH_3), 0.99, 0.97 (2s, 3H, $2'\text{CCH}_3$), 0.91, 0.89 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

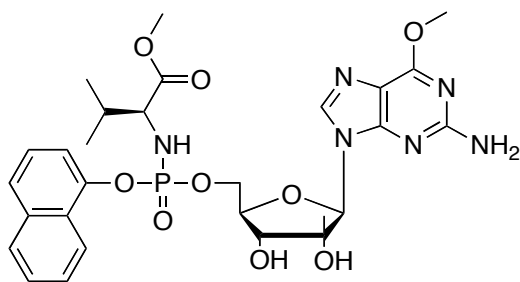
^{13}C NMR (126 MHz, MeOD) δ 174.87, 174.72 (2d, $^3J_{\text{C-C-N-P}}$ = 3.8 Hz, C=O), 162.74, 162.72 (C_6), 161.87, 161.84 (C_2), 157.05, 157.00 (ipso Ph) 154.50, 154.44 (C_4), 145.98 ($\text{C}_4\text{-Ph}$), 139.32, 139.01 (C_8), 126.54, 126.53 (C_3 and $\text{C}_5\text{-Ph}$), 122.22, 122.18 (C_2 and $\text{C}_6\text{-Ph}$), 115.61, 115.57 (C_5), 93.33, 93.13 ($\text{C}_{1'}$), 82.13, 81.98 (2d, $^3J_{\text{C-C-O-P}}$ = 7.6 Hz, C_4'), 80.05, 79.98 ($\text{C}_{2'}$), 75.48, 75.28 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 74.89, 74.54 ($\text{C}_{3'}$), 68.17, 67.54 (2d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C_5'), 54.31, 54.29 (6OCH_3), 51.64 (CHCH_3), 32.34, 32.28 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 26.81, 26.77, 26.74 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 20.80, 20.61 (2d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, CHCH_3), 20.42, 20.29 ($2'\text{CCH}_3$).

HPLC (System 1) t_{R} = 20.31, 20.16 min.

MS (ES+) m/z : 676.21 (MNa^+ , 100%).

HRMS calculated for $\text{C}_{26}\text{H}_{37}\text{N}_7\text{O}_{11}\text{P}_1$: 654.2289; found 654.2313.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(methoxy-L-valinyl)] phosphate (5.12l**).**



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(methoxy-L-valinyl) phosphorochloridate

(**3.3r**, 670 mg, 1.61 mmol), $t\text{BuMgCl}$ (1.60 ml, 1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (95:5), to give a pure product **5.12l** as a white foam (78.4 mg, 15%).

^{31}P NMR (202 MHz, MeOD) δ 5.15, 4.95.

^1H NMR (500 MHz, MeOD) δ 8.19 – 8.15 (m, 1H, H₈-Naph), 8.00 (s, 1H, H₈), 7.90-7.82 (m, 1H, H₅-Naph), 7.68, 7.65 (2d, $J = 7.7$ Hz, 1H, H₄-Naph), 7.57–7.43 (m, 3H, H₇, H₆, H₂-Naph), 7.40-7.34 (m, 1H, H₃-Naph), 6.01, 6.00 (2s, 1H, H_{1'}), 4.64- 4.61 (m, 2H, H_{5'}), 4.41- 4.26 (m, 2H, H_{3'} and H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.76 – 3.72 (m, 1H, CHCH(CH₃)₂), 3.53, 3.50 (2s, 3H, OCH₃), 2.03- 1.92 (m, 1H, CHCH(CH₃)₂), 1.00, 0.96 (2s, 3H, 2'CCH₃), 0.88- 0.79 (m, 6H, CHCH(CH₃)₂).

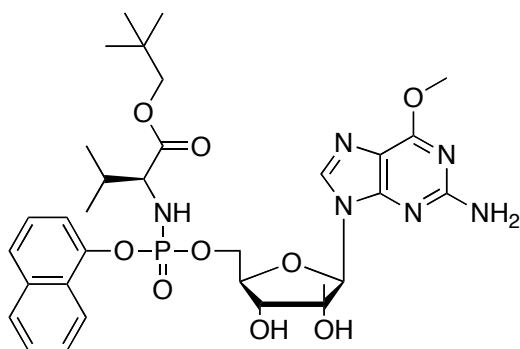
^{13}C NMR (126 MHz, MeOD) δ 174.74, 174.41 (2d, $^3J_{\text{C-C-N-P}} = 2.5$ Hz, C=O), 162.64 (C6), 161.88, 161.87 (C2), 154.39 (C4), 148.03, 147.97 (ipso Naph), 139.33, 139.07 (C8), 136.27, 136.22 (C10-Naph), 128.81, 128.75, 128.52, 127.87, 127.82, 127.74, 127.70, 127.41, 126.56, 126.50, 126.43, 125.90, 122.79 (Naph), 116.16, 116.14 (C2-Naph), 115.38, 115.30 (C5), 93.47, 93.33 (C1'), 82.37, 82.23 (2d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C4'), 79.93, 79.91 (C2'), 74.88, 74.75 (C3'), 68.18, 67.70 (d, $^2J_{\text{C-O-P}} = 6.3$ Hz, C5'), 61.97, 61.92 (OCH₃ ester) 54.31, 54.29 (6OCH₃), 52.43, 52.39, 52.03 (CHCH(CH₃)₂), 33.27, 33.03 (2d, $^3J_{\text{C-C-N-P}} = 8.8$ Hz, CHCH(CH₃)₂), 20.35, 20.32 (2'CCH₃), 19.45, 19.42, 18.45, 18.39 (CHCH(CH₃)₂).

HPLC (System 1) $t_R = 18.28$ min.

MS (ES+) m/z : 631.23 (MH⁺, 100%).

HRMS calculated for C₂₈H₃₆N₆O₉P₁: 631.2281; found 631.2252.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2dimethylpropoxy-L-valinyl)] phosphate (5.12m).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 150 mg, 0.48 mmol), α -naphthyl-(methoxy-L-valinyl) phosphorochloridate (**3.3s**, 393 mg, 0.97 mmol), *t*BuMgCl (0.97 ml, 0.97 mmol) in 5 ml of dry THF.

The crude mixture was purified by column chromatography, using CHCl₃/MeOH (97:3), to give a pure product **5.12m** as a white foam (46 mg, 14%).

^{31}P NMR (202 MHz, MeOD) δ 5.10, 4.95.

^1H NMR (500 MHz, MeOD) δ 8.19–8.15 (m, 1H, H₈-Naph), 8.02 (s, 1H, H₈) 7.88–7.82 (m, 1H, H₅-Naph), 7.68, 7.65 (2d, J = 8.5 Hz, 1H, H₄-Naph), 7.52–7.43 (m, 3H, H₇, H₆, H₂-Naph), 7.39, 7.36 (2t, 1H, J = 8.0 Hz, H₃-Naph), 6.00, 5.99 (2s, 1H, H_{1'}), 4.64–4.60 (m, 2H, H_{5'}), 4.37–4.24 (m, 2H, H_{3'} and H_{4'}), 4.05 (s, 3H, 6OCH₃), 3.82–3.75 (m, 1H, CHCH(CH₃)₂), 3.91–3.81 (m, 2H, OCH₂C(CH₃)₃), 2.08–.95 (m, 1H, CHCH(CH₃)₂), 0.99, 0.96 (2s, 3H, 2'CCH₃), 0.89–0.83 (m, 15H, CHCH(CH₃)₂ and OCH₂C(CH₃)₃).

^{13}C NMR (126 MHz, MeOD) δ 174.34, 174.09 (2d, $^3J_{\text{C-C-N-P}}$ = 2.5 Hz, C=O), 162.59, 162.55 (C6), 161.91, 161.88 (C2), 154.26 (C4), 148.02, 147.97 (ipso Naph), 139.36, 139.07 (C8), 136.29, 136.23 (C10-Naph), 128.83, 128.76 (CH-Naph), 127.86, 127.71 (2d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C9-Naph), 127.42, 126.51, 126.44, 125.90, 122.83, 122.81 (CH-Naph), 116.19, 116.13 (2d, $^3J_{\text{C-C-O-P}}$ = 3.8 Hz, C2-Naph), 115.15, 115.00 (C5), 93.55, 93.40 (C1'), 82.46, 82.31 (2d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C4'), 79.91, 79.88 (C2'), 75.50, 75.48 (OCH₂C(CH₃)₃), 74.94, 74.81 (C3'), 68.34, 67.88 (2d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C5'), 62.05 (6OCH₃) 54.36, 54.33 (CHCH(CH₃)₂), 33.31, 33.11 (2d, $^3J_{\text{C-C-N-P}}$ = 8.8 Hz, CHCH(CH₃)₂), 32.05, 32.00 (OCH₂C(CH₃)₃), 26.80, 26.78, 26.74 (OCH₂C(CH₃)₃), 20.34, 20.31 (2'CCH₃), 18.32, 18.20 (CHCH(CH₃)₂).

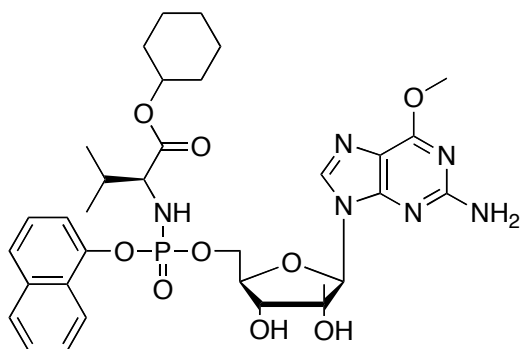
HPLC (System 1) t_{R} = 21.32 min.

HPLC (System 2) t_{R} = 29.16, 29.75 min.

MS (ES+) m/z : 687.29 (MH⁺, 100%).

HRMS calculated for C₃₂H₄₄N₆O₉P₁: 687.2907 found 687.2888

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(cyclohexoxy-L-valinyl)] phosphate (5.12n).



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (5.4, 150 mg, 0.482 mmol), α -naphthyl-(cyclohexoxy-L-valinyl) phosphorochloridate (633 mg, 1.44 mmol, 1M solution in THF), NMI (0.19 ml, 2.41

mmol) in 5 ml of dry THF. The crude mixture was purified by column

chromatography, using $\text{CHCl}_3/\text{MeOH}$ (97:7) as eluent, to give a pure product **5.12o** as a white foam (45 mg, 11%).

^{31}P NMR (202 MHz, MeOD) δ 5.15, 4.91.

^1H NMR (500 MHz, MeOD) δ 8.21–8.14 (m, 1H, $\text{H}_8\text{-Naph}$), 7.96, 7.94 (s, 1H, H_8), 7.89–7.82 (m, 1H, $\text{H}_5\text{-Naph}$), 7.69, 7.65 (2d, $J = 8.5$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.55–7.42 (m, 3H, H_7 , H_6 , $\text{H}_2\text{-Naph}$), 7.42–7.33 (m, 1H, $\text{H}_3\text{-Naph}$), 5.99 (s, 1H, $\text{H}_{1'}$), 4.66–4.54 (m, 3H, OCH ester, $\text{H}_{5'}$), 4.39–4.22 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{4'}$), 4.05, 4.04 (2s, 3H, 6OCH_3), 3.75–3.68 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 2.06–1.93 (m, 1H, $\text{CHCH}(\text{CH}_3)_2$), 1.70–1.57 (m, 4H, 2x CH_2 ester), 1.39–1.15 (m, 6H, 3x CH_2 ester), 0.99, 0.95 (s, 3H, $2'\text{CCH}_3$), 0.89 – 0.82 (m, 6H, $\text{CHCH}(\text{CH}_3)_2$).

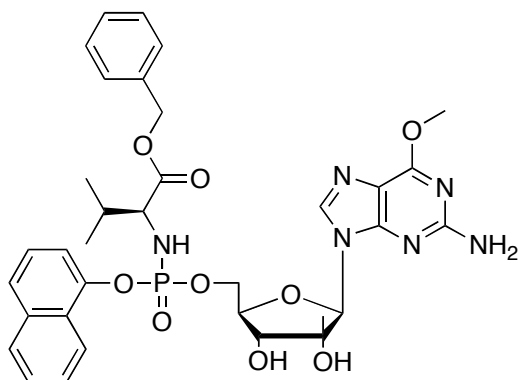
^{13}C NMR (126 MHz, MeOD) δ 173.67 (d, $^3J_{\text{C-C-N-P}} = 2.8$ Hz, C=O), 173.39 (d, $^3J_{\text{C-C-N-P}} = 3.4$ Hz, C=O), 162.73 (C_6), 161.89, 161.86 (C_2), 154.53, 154.49 (C_4), 148.06, 148.00 (2d, $^2J_{\text{C-O-P}} = 1.6$, ipso Naph), 139.44, 139.15 (C_8), 136.30, 136.25 ($\text{C}_{10}\text{-Naph}$), 128.81, 128.75 (CH-Naph), 127.88, 127.86 (2d, $^3J_{\text{C-C-O-P}} = 6.5$ Hz, Naph- C_9), 127.72, 127.67, 127.39, 126.49, 126.42, 125.85, 122.85, 122.83 (CH-Naph), 116.12, 116.07 (2d, $^3J_{\text{C-C-O-P}} = 3.2$, $\text{C}_2\text{-Naph}$), 115.67, 115.62 (C_5), 93.48, 93.29 ($\text{C}_{1'}$), 82.41, 82.22 (2d, $^3J_{\text{C-C-O-P}} = 8.0$ Hz, C_4'), 79.89 (C_2'), 74.98, 74.93 (C_3'), 74.86, 74.81 (OCH ester), 68.36, 67.82 (2d, $^2J_{\text{C-O-P}} = 5.5$ Hz, C_5'), 62.05, 62.01 ($\text{CHCH}(\text{CH}_3)_2$), 54.23, 54.22 (6OCH_3), 33.34 (d, $^3J_{\text{C-C-N-P}} = 6.9$ Hz, $\text{CHCH}(\text{CH}_3)_2$), 33.16 (d, $^3J_{\text{C-C-N-P}} = 7.4$, $\text{CHCH}(\text{CH}_3)_2$), 32.46, 32.39 (CH_2 ester), 26.35 (CH_2 ester), 24.62, 24.58 (CH_2 ester), 20.32, 20.28 ($2'\text{CCH}_3$), 19.46, 19.46, 19.44 ($\text{CHCH}(\text{CH}_3)_2$), 18.35, 18.23 ($\text{CHCH}(\text{CH}_3)_2$).

HPLC (System 1) $t_R = 21.61, 22.11$ min.

MS (ES+) m/z : 721.27 (MNa^+ , 100%).

HRMS calculated for $\text{C}_{33}\text{H}_{43}\text{N}_6\text{O}_9\text{P}_1\text{Na}$: 721.2730; found 721.2727.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-valinyl)] phosphate (5.12o).



Prepared according to the Standard Procedure 6 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3t**, 1.04 g, 2.41 mmol), NMI (0.32 ml, 4.02 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (97:3) as eluent to give a pure product **5.12p** as a white foam (170 mg, 30%).

³¹P NMR (202 MHz, MeOD) δ 5.08, 5.00.

¹H NMR (500 MHz, MeOD) δ 8.17- 8.15 (m, 1H, H₈-Naph), 7.98, 7.96 (2s, 1H, H₈), 7.86- 7.82 (m, 1H, H₅-Naph), 7.68- 7.64 (m, 1H, H₄-Naph), 7.52-7.41 (m, 3H, H₇, H₆, H₂-Naph), 7.37, 7.36 (2t, J = 8.0 Hz, 1H, H₃-Naph), 7.25-7.21 (m, 5H, OCH₂Ph), 5.03, 4.93 (AB, J_{AB} = 12.0 Hz, 2H, OCH₂Ph), 4.61 - 4.58 (m, 2H, H_{5'}), 4.34, 4.27 (2d, J = 9.0 Hz, 1H, H_{3'}), 4.25- 4.21 (m, 1H, H_{4'}), 4.04, 4.03 (2s, 1H, 6OCH₃), 3.81- 3.76 (m, 1H, CHCH(CH₃)₂), 2.04 - 1.91 (m, 1H, CHCH(CH₃)₂), 0.98, 0.94 (2s, 3H, 2'CCH₃), 0.82- 0.78 (m, 6H, CHCH(CH₃)₂).

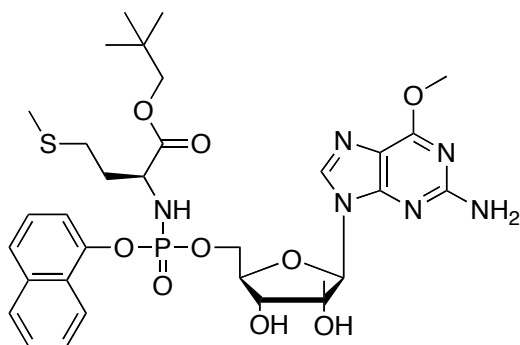
¹³C NMR (126 MHz, MeOD) δ 174.04, 173.78 (2d, $^3J_{C-C-N-P}$ = 2.5 Hz, C=O), 162.64 (C6), 161.85 (C2), 154.38 (C4), 148.02, 147.97 (ipso Naph), 139.37, 139.05 (C8), 137.11, 137.08 (ipso OCH₂Ph), 137.06 (C10-Naph), 136.23, 129.48, 129.43, 129.40, 129.28, 128.82, 128.76, 127.90, 127.85, 127.74, 127.70, 127.44, 127.24, 126.76, 126.63, 126.51, 126.44, 126.34, 125.92, 124.02, 123.53, 122.85, 122.81 (Naph, OCH₂Ph), 116.15, 116.16 (2d, $^3J_{C-C-O-P}$ = 3.8 Hz, C2-Naph), 115.40, 115.31 (C5), 93.50, 93.28 (C1'), 82.41, 82.21 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.91, 79.88 (C2'), 74.95, 74.71 (C3'), 68.32, 68.28 (OCH₂Ph), 67.81, 67.65 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 62.02, 61.96 (6OCH₃) 54.32, 54.28 (CHCH(CH₃)₂), 33.31, 33.11 (2d, $^3J_{C-C-N-P}$ = 8.8 Hz, CHCH(CH₃)₂), 20.35, 20.32 (2'CCH₃), 19.46, 19.43, 19.31, 18.42, 18.36, 18.22 (CHCH(CH₃)₂).

HPLC t_R = 21.64 min.

MS (ES+) m/z : 707.26 (MH^+ , 100%).

HRMS calculated for $C_{34}H_{40}N_6O_9P_1$: 707.2594; found 707.2572.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-methioninyl)] phosphate (5.12p).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-methioninyl) phosphorochloridate (**3.3y**, 713 mg, 1.61 mmol, 1M solution in THF), *t*BuMgCl

(1.61 ml, 1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using $CHCl_3/MeOH$ (0 to 5%, gradient), to give a pure product **5.12p** as a white foam (214 mg, 37%).

^{31}P NMR (202 MHz, MeOD) δ 4.50, 4.44.

1H NMR (500 MHz, MeOD) δ 8.23 – 8.16 (m, 1H, H_8 -Naph), 7.99, 7.97 (2s, 1H, H_8), 7.88 – 7.8. (m, 1H, H_5 -Naph), 7.69, 7.66 (2d, J = 8.6 Hz, 1H, H_4 -Naph), 7.55 – 7.45 (m, 3H, H_7 , H_6 , H_2 -Naph), 7.41, 7.37 (2t, J = 8.0 Hz, 1H, H_3 -Naph), 6.01, 6.00 (2s, 1H, $H_{1'}$), 4.72 – 4.59 (m, 2H, $H_{5'}$), 4.35 – 4.23 (m, 2H, $H_{3'}$ and $H_{4'}$), 4.18- 4.13 (m, 1H, $CHCH_2CH_2SCH_3$), 4.05, 4.04 (2s, 3H, $6OCH_3$), 3.75, 3.71, 3.66, 3.62 (2AB, J_{AB} = 10.0 Hz, 2H, $OCH_2C(CH_3)_3$), 2.47-2.38, 2.32-2.29 (2m, 2H, $CHCH_2CH_2SCH_3$), 2.02-1.81 (m, 5H, $CHCH_2CH_2SCH_3$ and $CHCH_2CH_2SCH_3$), 0.98, 0.97 (2s, 3H, $2'CCH_3$), 0.88, 0.85 (2s, 9H, $OCH_2C(CH_3)_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.52, 174.28 (d, $^3J_{C-C-N-P}$ = 3.8, C=O), 162.7 (C6), 161.93, 161.87 (C2), 154.57, 154.51 (C4), 148.00, 147.95 (2d, $^2J_{C-O-P}$ = 6.3 Hz, ipso Naph), 139.41, 139.29 (C8), 136.31, 136.25 (C10-Naph), 128.94, 128.87 (CH-Naph), 127.95 (d, $^3J_{C-C-O-P}$ = 3.8 Hz, C9-Naph), 127.88 (d, $^3J_{C-C-O-P}$ = 5.0 Hz, C9-Naph), 127.72, 127.63, 127.55, 126.51, 126.09, 122.89 (CH-Naph), 116.32 (d, $^3J_{C-C-O-P}$ = 3.8, C2-Naph), 116.16 (d, $^3J_{C-C-O-P}$ = 2.5, C2-Naph), 115.71, 115.68 (C5), 93.35, 93.22

(C1'), 82.34 (d, $^3J_{C-C-O-P} = 7.5$, C4'), 82.22 (d, $^3J_{C-C-O-P} = 8.8$, C4'), 80.00, 79.95 (C2'), 75.63 (OCH₂C(CH₃)₃), 74.9, 74.8 (C3'), 68.27, 67.82 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 55.27, 55.19 (CHCH₂CH₂SCH₃), 54.21, 54.09 (OCCH₃), 34.52, 34.35 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₂CH₂SCH₃), 32.21, 32.17 (OCH₂C(CH₃)₃), 31.02, 30.94 (CHCH₂CH₂SCH₃), 26.91, 28.82, 28.75 (OCH₂C(CH₃)₃), 20.42, 20.38 (2'CCH₃), 15.22, 15.16 (CHCH₂CH₂SCH₃).

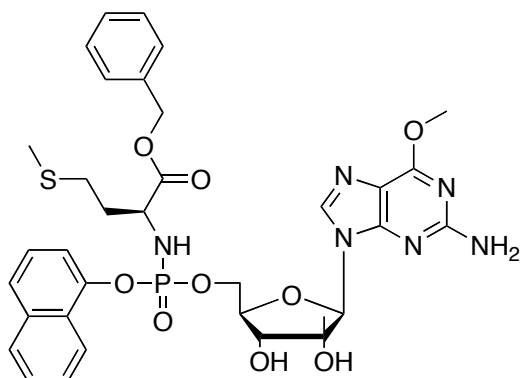
HPLC (System 1) t_R = 24.36 min.

HPLC (System 1) t_R = 27.89, 28.76 min.

MS (ES+) m/z : 719.26 (MH⁺, 100%).

HRMS calculated for C₃₂H₄₄N₆O₉P₁: 719.2628; found 719.2645.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(benzoxy-L-methioninyl)] phosphate (5.12q).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α-naphthyl-(benzoxy-L-methioninyl) phosphorochloridate (**3.3z**, 742 mg, 1.61 mmol, 1M solution in THF), *t*BuMgCl

(1.61 ml, 1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product **5.12p** as a white foam (246 mg, 42%).

³¹P NMR (202 MHz, MeOD) δ 4.54, 4.42

¹H NMR (500 MHz, MeOD) δ 8.19, 8.16 (2d, $J = 7.8$ Hz, 1H, H₈-Naph), 7.98, 7.94 (2s, 1H, H₈) 7.86, 7.84 (2d, $J = 9.0$ Hz, 1H, H₅-Naph), 7.68, 7.66 (2d, $J = 8.2$ Hz, 1H, H₄-Naph), 7.53–7.44 (m, 3H, H₇, H₆, H₂-Naph), 7.36, 7.35 (2t, $J = 8.0$ Hz, 1H, H₃-Naph), 7.26–7.25 (m, 5H, OCH₂Ph), 6.00, 5.99 (2s, 1H, H_{1'}), 5.08–4.95 (m, 2H, OCH₂Ph), 4.66–4.58 (m, 2H, H_{5'}), 4.35, 4.29 (2d, $J = 9.0$ Hz, 1H, H_{3'}), 4.26–4.22 (m, 1H, H_{4'}), 4.18–4.13 (m, 1H, OCH₂C(CH₃)₃), 4.03, 4.02 (2s, 3H, 6OCH₃), 2.35–2.24

(m, 2H, CHCH₂CH₂SCH₃), 1.98-1.77 (m, 5H, CHCH₂CH₂SCH₃ and CHCH₂CH₂SCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃).

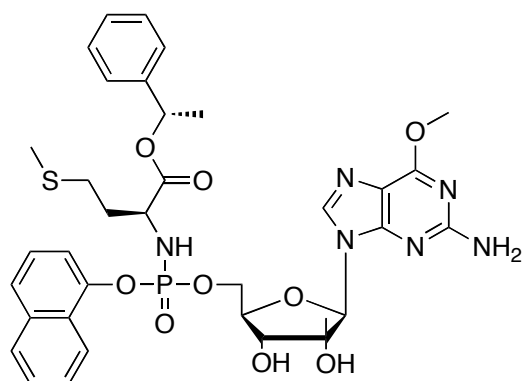
¹³C NMR (126 MHz, MeOD) δ 174.21, 173.89 (2d, ³J_{C-C-N-P} = 3.8 Hz, C=O), 162.72 (C6), 161.85 (C2), 154.56, 154.51 (C4), 148.00, 147.94 (ipso Naph), 139.34, 139.09 (C8), 137.08, 137.06 (ipso OCH₂Ph), 136.28, 136.25 (C10-Naph), 129.53, 129.36, 129.31, 128.85, 128.83 (Naph, OCH₂Ph), 127.89 (d, ³J_{C-C-O-P} = 3.8 Hz, C9-Naph), 127.80 (d, ³J_{C-C-O-P} = 5.0 Hz, C9-Naph), 127.54, 127.52, 126.52, 126.03, 125.97, 122.86, 122.82 (Naph, OCH₂Ph), 116.35, 116.25 (d, ³J_{C-C-O-P} = 3.8 Hz, C2-Naph), 116.15 (d, ³J_{C-C-O-P} = 2.5 Hz, C2-Naph), 115.64, 115.60 (C5), 93.35, 93.16 (C1'), 82.32, 82.13 (2d, ³J_{C-C-O-P} = 8.8 Hz, C4'), 79.98, 79.93 (C2'), 74.91, 74.63 (C3'), 68.17 (d, ²J_{C-O-P} = 5.0 Hz, C5'), 68.04 (d, ²J_{C-O-P} = 6.25 Hz, C5'), 67.59, 67.55 (OCH₂Ph), 55.23, 55.11 (CHCH₂CH₂SCH₃), 54.26 (6OCH₃), 34.35 (d, ³J_{C-C-N-P} = 6.25 Hz, CHCH₂CH₂SCH₃), 34.13 (d, ³J_{C-C-N-P} = 7.5 Hz, CHCH₂CH₂SCH₃), 30.74, 30.72 (CHCH₂CH₂SCH₃), 20.36 (2'CCH₃), 15.13, 15.09 (CHCH₂CH₂SCH₃).

HPLC t_R = 19.13, 19.59 min.

MS (ES+) m/z: 761.20 (MNa⁺, 100%).

HRMS calculated for C₃₄H₃₉N₆O₉P₁Na: 761.2135; found 761.2097.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(*S*-phenylethoxy-L-methioninyl)] phosphate (5.12r).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α-naphthyl-(*S*-phenylethoxy-L-methioninyl) phosphorochloridate (**3.3za**, 768 mg, 1.61

mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (97:3), to give a pure product **5.12r** as a white foam (226 mg, 37%).

³¹P NMR (202 MHz, MeOD) δ 4.53, 4.44.

^1H NMR (500 MHz, MeOD) δ 8.22–8.17 (m, 1H, H₈-Naph), 7.99, 7.96 (2s, 1H, H₈) 7.86 – 7.82 (m, 1H, H₅-Naph), 7.67, 7.65 (2d, J = 8.5 Hz, 1H, H₄-Naph), 7.55–7.46 (m, 3H, H₇, H₆, H₂-Naph), 7.37, 7.35 (2t, J = 8.0 Hz, 1H, H₃-Naph), 7.29–7.26 (m, 5H, OCH(CH₃)Ph), 6.03, 6.01 (2s, 1H, H_{1'}), 5.77, 5.69 (2q, J = 5.0 Hz, OCH(CH₃)Ph), 4.68–4.54 (m, 2H, H_{5'}), 4.35, 4.31 (2d, J = 9.0 Hz, 1H, H_{3'}), 4.28–4.23 (m, 1H, H_{4'}), 4.16–4.12 (m, 1H, CHCH₂CH₂SCH₃), 4.04, 4.03 (2s, 3H, 6OCH₃), 2.33–2.11 (m, 1H, CHCH₂CH₂SCH₃), 1.93–1.86, 1.80–1.72 (2m, 2H, CHCH₂CH₂SCH₃), 1.82, 1.81 (2s, 3H, CHCH₂CH₂SCH₃), 1.40, 1.39 (2d, J = 5.0 Hz, 3H, OCH(CH₃)Ph), 0.98, 0.97 (2s, 3H, 2'CCH₃).

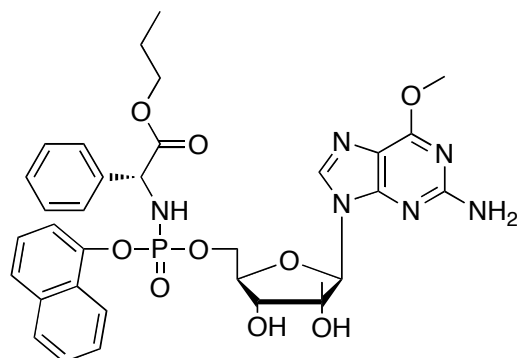
^{13}C NMR (126 MHz, MeOD) δ 173.63, 173.33 (2d, $^3J_{\text{C-C-N-P}}$ = 3.8 Hz, C=O), 162.75 (C6), 161.87 (C2), 154.58, 154.54 (C4), 147.98, 147.95 (2d, $^2J_{\text{C-O-P}}$ = 6.3 Hz, ipso Naph), 139.42, 139.12 (C8), 137.68 (ipso OCH(CH₃)Ph), 136.29, 136.25 (C10-Naph), 129.68, 129.57, 129.55, 129.08, 128.88, 128.86, (CH-Naph, OCH(CH₃)Ph), 127.89 (d, $^3J_{\text{C-C-O-P}}$ = 3.8 Hz, naph-C9), 127.79 (d, $^3J_{\text{C-C-O-P}}$ = 3.8 Hz, C9-Naph), 127.55, 127.53, 127.25, 126.53, 126.00, 122.86, 122.83 (CH-Naph, OCH(CH₃)Ph), 116.27, 116.25 (C2-Naph), 115.67, 115.62 (C5), 93.37, 93.17 (C1'), 82.36 (d, $^3J_{\text{C-C-O-P}}$ = 7.5 Hz, C4'), 82.15 (d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C4'), 80.00, 79.94 (C2'), 75.03, 74.96 (C3'), 74.92, 74.70 (OCH(CH₃)Ph), 68.26, 67.66 (2d, $^2J_{\text{C-O-P}}$ = 6.25 Hz, C5'), 55.20, 55.12 (CHCH₂CH₂SCH₃), 55.02, 54.32 (6OCH₃), 34.34, 34.17 (2d, $^3J_{\text{C-C-N-P}}$ = 7.5 Hz, CHCH₂CH₂SCH₃), 30.65, 30.61 (CHCH₂CH₂SCH₃), 22.47, 22.41 (OCH(CH₃)Ph) 20.40, 20.38 (2'CCH₃), 15.13, 15.11 (CHCH₂CH₂SCH₃).

HPLC t_{R} = 20.87, 21.35 min.

MS (ES+) m/z : 775.23 (MNa⁺, 100%).

HRMS calculated for C₃₅H₄₁N₆O₉P₁Na: 775.2291; found 775.2271.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(propoxy-D-phenylglycyl)] phosphate (5.12s).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(propoxy-D-phenylglycyl) phosphorochloridate (**3.3zb**, 670 mg, 1.61

mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.12s** was obtained as a white foam (395 mg of 71%).

³¹P NMR (202 MHz, MeOD) δ 4.38, 3.68.

¹H NMR (500 MHz, MeOD) δ 8.00–7.96 (m, 2H, H₈-Naph and H₈), 7.84, 7.81 (2d, *J* = 8.0 Hz, 1H, H₅-Naph), 7.65, 7.62 (2d, *J* = 8.5 Hz, 1H, H₄-Naph), 7.50–7.37 (m, 3H, H₂, H₇, H₆-Naph), 7.31–7.30 (m, 1H, H₃-Naph), 7.28–7.18 (m, 5H, CHPh), 6.01, 5.99 (2s, 1H, H_{1'}), 5.05, 5.01 (2d, *J* = 9.0 Hz, 1H, CHPh), 4.66–4.53 (m, 2H, H_{5'}), 4.36, 4.29 (2d, *J* = 9.0 Hz, 1H, H_{3'}), 4.27–4.20 (m, 1H, H_{4'}), 4.06 (s, 3H, 6OCH₃), 3.98–3.88 (m, 2H, OCH₂CH₂CH₃), 1.49 - 1.39 (m, 2H, OCH₂CH₂CH₃), 0.98, 0.97 (2s, 3H, 2'CCH₃), 0.71, 0.69 (2t, *J* = 7.5 Hz, 3H, OCH₂CH₂CH₃).

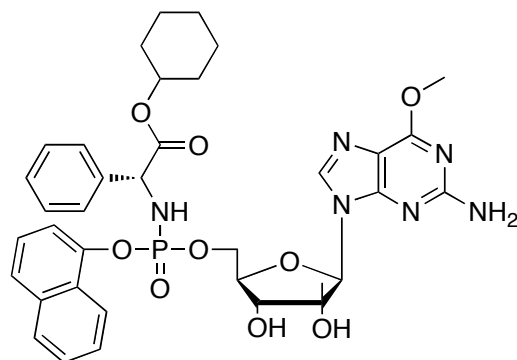
¹³C NMR (126 MHz, MeOD) δ 173.05, 172.75 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 162.77, 162.70 (C6), 161.91, 161.85 (C2), 154.65, 154.50 (C4), 147.89, 147.84 (ipso Naph), 139.45, 139.09 (C8), 139.29 (d, ³*J*_{C-C-N-P} = 6.30 Hz, CHPh), 139.20 (d, ³*J*_{C-C-N-P} = 5.0 Hz, ipso CHPh), 136.23, 136.20 (C10-Naph), 129.61, 129.19, 128.72, 128.69, 128.16, 128.14, (CH-Naph and CHPh), 127.86, 127.76 (2d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.67, 127.43, 127.37, 126.40, 126.34, 125.93, 125.89, 122.82, 122.76 (CH-Naph and CHPh), 116.25, 115.95 (2d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 115.64, 115.56 (C5), 93.40, 93.08 (C1'), 82.32 (d, ³*J*_{C-C-O-P} = 7.6 Hz, C4'), 82.00 (d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 79.97, 79.93 (C2'), 74.96, 74.60 (C3'), 68.24 (d, ²*J*_{C-O-P} = 2.5 Hz, C5'), 68.15 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 67.29, 67.25 (OCH₂CH₂CH₃), 59.93, 59.73 (d, ²*J*_{C-N-P} = 5.0 Hz, C5', CHPh), 54.27, 54.22 (6OCH₃), 22.80, 22.77 (OCH₂CH₂CH₃), 20.33, 20.28 (2'CCH₃), 10.43, 10.41 (OCH₂CH₂CH₃).

HPLC (System 1) t_R = 16.91 min

MS (ES+) m/z : 715.23 (MNa^+ , 100%).

HRMS calculated for $C_{33}H_{38}N_6O_9P_1$: 693.2438; found 693.2463.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(cyclohexoxy-D-phenylglyciny)] phosphate (5.12t).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), α -naphthyl-(propoxy-D-phenylglyciny) phosphorochloridate (**3.3zc**, 735 mg, 1.61

mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF. After silica gel column chromatography, using $CHCl_3/MeOH$ (0 to 5%, gradient) as eluent, pure product **5.12t** was obtained as a white foam (360 mg of 61%).

^{31}P NMR (202 MHz, MeOD) δ 4.41, 3.70.

1H NMR (500 MHz, MeOD) δ 8.00–7.95 (m, 2H, H_8 -naph and H_8), 7.83, 7.80 (2d, J = 8.0 Hz 1H, H_5 -Naph), 7.64, 7.62 (2d, J = 8.0 Hz, 1H, H_4 -Naph), 7.50–7.38 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.33 - 7.18 (m, 6H, H_3 -Naph and $CHPh$), 6.02, 5.99 (2s, 1H, $H_{1'}$), 5.03, 4.99 (2d, J = 9.0 Hz, 1H, $CHPh$), 4.67–4.55 (m, 3H, OCH ester and $H_{5'}$), 4.36–4.27 (m, 2H, $H_{3'}$, $H_{4'}$), 4.06 (s, 3H, 6OCH₃), 1.68–1.49 (m, 4H, 2x CH₂ ester), 1.40–1.28 (m, 4H, 2x CH₂ ester), 1.18–1.13 (m, 2H, CH₂ ester), 0.97, 0.97 (2s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, MeOD) δ 172.40, 172.09 (2d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.70, 162.70 (C6), 161.91, 161.84 (C2), 154.65, 154.51 (C4), 147.90, 147.86 (ipso Naph), 139.44, 139.03 (C8), 139.33, 139.29 (ipso $CHPh$), 136.24, 136.21 (C10-Naph), 129.79, 129.60, 129.15, 128.74, 128.70, 128.16, 128.14 (CH-Naph and $CHPh$), 127.86, 127.77 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.67, 127.44, 127.37, 126.42, 126.35, 125.93, 125.89, 122.84, 122.77 (CH-Naph and $CHPh$), 116.24, 115.94 (2d, $^3J_{C-C-O-P}$ = 3.8 Hz, C2-Naph), 115.65, 115.57 (C5), 93.39, 93.05 (C1'), 82.32, 81.98 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.98, 79.96 (C2'), 75.26, 75.20 (OCH ester), 74.97,

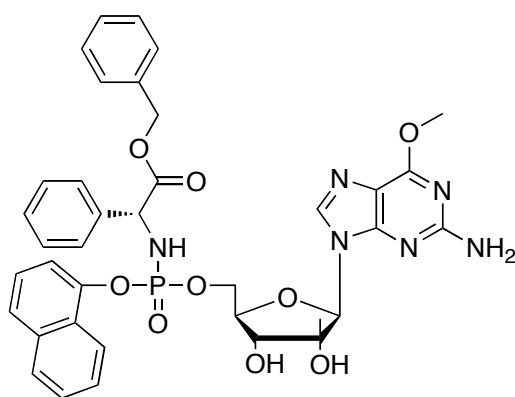
74.78 (C3'), 68.16, 67.25 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 60.02, 59.83 (d, $^2J_{C-N-P} = 3.5$ Hz, CHPh), 54.30, 54.24 (6OCH₃), 32.37, 32.23, 32.19, 32.00 (CH₂ ester), 26.37, 26.29 (CH₂ ester), 24.29, 24.21 (CH₂ ester), 20.36, 20.30 (2'CCH₃)

HPLC (System 1) $t_R = 18.40$ min.

MS (ES+) m/z : 755.26 (MNa⁺, 100%).

HRMS calculated for C₃₆H₄₂N₆O₉P₁Na: 755.2570; found 755.2576.

Synthesis of 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(benzoxy-*D*-phenylglyciny)] phosphate (5.12u).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (5.4, 250 mg, 0.80 mmol), α-naphthyl-(benzoxy-*D*-phenylglyciny) phosphorochloridate (3.3zd, 735 mg, 1.61 mmol), *t*BuMgCl (1.61 ml, 1.61 mmol) in 5 ml of dry THF. After silica gel column

chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product 5.12u was obtained as a white foam (360 mg of 48%).

³¹P NMR (202 MHz, MeOD) δ 4.37, 3.65.

¹H NMR (500 MHz, MeOD) δ 8.02, 7.99 (2d, $J = 7.5$ Hz, 1H, H₈-Naph), 7.95, 7.94 (2s, 1H, H₈) 7.83, 7.81 (2d, $J = 8.5$ Hz, 1H, H₅-Naph), 7.65, 7.62 (2d, $J = 8.0$ Hz, 1H, H₄-Naph), 7.49–7.06 (m, 14H, H₇, H₆, H₂, H₃-Naph, OCH₂Ph and CHPh), 5.99, 5.98 (2s, 1H, H_{1'}), 5.15 – 5.04 (m, 2H, OCH₂Ph), 5.00, 4.97 (2d, $J = 5.0$ Hz, 1H, CHPh), 4.65–4.51 (m, 2H, H_{5'}), 4.35–4.21 (m, 2H, H_{3'} and H_{4'}), 4.05, 4.04 (2s, 3H, 6OCH₃), 0.97, 0.95 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 172.81, 172.55 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, C=O), 162.75, 162.71 (C6), 161.81 (C2), 154.59, 154.49 (C4), 147.87, 147.82 (ipso Naph), 139.44, 139.98 (C8), 138.90 (CHPh), 136.82, 136.76 (C10-Naph), 136.18 (OCH₂Ph), 129.85, 129.69, 129.47, 129.41, 129.30, 129.16, 129.14, 128.98, 128.89, 128.83, 128.75, 128.26, 127.84, 127.78, 127.71, 127.52, 127.43, 126.45, 126.40, 126.00, 125.96, 122.85, 122.76 (Naph, OCH₂Ph and CHPh), 116.37 (d, $^3J_{C-C-O-P} = 3.8$ Hz, C2-Naph),

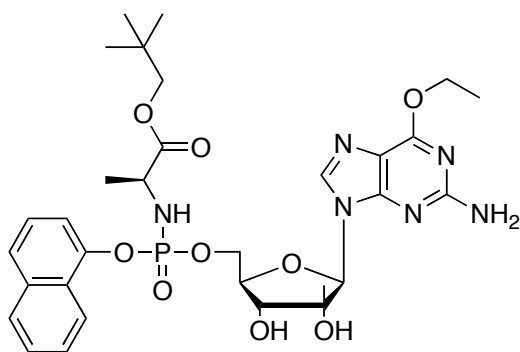
116.03 (d, $^3J_{C-C-O-P} = 2.5$ Hz, C2-Naph), 115.72, 115.65 (C5), 93.38, 93.06 (C1'), 82.32 (d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 81.98 (d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 80.02 (C2'), 75.00, 74.56 (C3'), 68.24 (d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 68.15 (d, $^2J_{C-O-P} = 6.3$ Hz, C5'), 67.24 (OCH₂Ph), 60.00, 59.79 (CHPh), 54.38 (6OCH₃), 20.48 (2'CCH₃).

HPLC (System 1) t_R = 19.49, 19.89 min.

MS (ES+) m/z : 763.21 (MNa⁺, 100%).

HRMS calculated for C₃₇H₃₈N₆O₉P₁Na: 741.2438; found 741.2465.

Synthesis of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.14a).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.5**, 250 mg, 0.77 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 589 mg, 1.54

mmol), *t*BuMgCl (1.54 ml, 1.54 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.14a** was obtained as a white foam (145 mg of 28%).

³¹P NMR (202 MHz, MeOD) δ 4.28, 4.21.

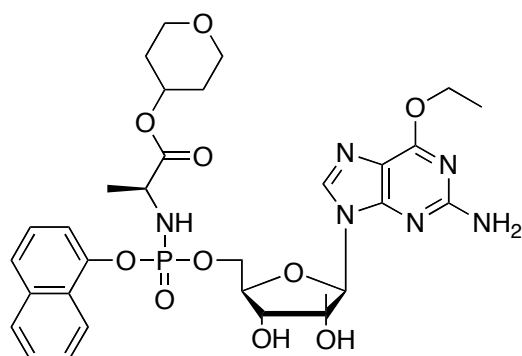
¹H NMR (500 MHz, MeOD) δ 8.20-8.14 (m, 1H, H₈-Naph), 7.96, 7.94 (2s, 1H, H₈), 7.89–7.84 (m, 1H, H₅-Naph), 7.71-7.68 (m, 1H, H₄-Naph), 7.58–7.46 (m, 3H, H₇, H₆, H₂-Naph), 7.43-7.36 (m, 1H, H₄-Naph), 5.99, 5.98 (2s, 1H, H_{1'}), 4.65- 4.55 (m, 2H, H_{5'}), 4.53, 4.51 (2q, $J=6.70$, 2H, 6OCH₂CH₃), 4.35- 4.22 (m, 2H, H_{3'} and H_{4'}), 4.08-3.99 (m, 1H, CHCH₃), 3.78-3.67 (m, 2H, OCH₂C(CH₃)₃), 1.44 (t, $J=7.20$ Hz, 3H, 6OCH₂CH₃), 1.33, 1.31 (2d, $J=6.35$ Hz, 3H, CHCH₃), 0.98, 0.97 (2s 3H, 2'CCH₃), 0.88, 0.86 (OCH₂C(CH₃)₃)

HPLC (System 1) t_R = 22.92, 23.16 min

MS (ES+) m/z : 695.26 (MNa⁺, 100%).

HRMS calculated for C₃₁H₄₂N₆O₉P₁Na: 695.2671; found 695.2624.

Synthesis of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(tetrahydropyroxyl-L-alaninyl)] phosphate (5.14b).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.5**, 250 mg, 0.77 mmol), α -naphthyl-(tetrahydropyroxyl-L-alaninyl) phosphorochloridate (**3.3j**, 612 mg, 1.54

mmol), *t*BuMgCl (1.54 ml, 1.54 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.14b** was obtained as a white foam (72 mg of 14%).

³¹P NMR (202 MHz, MeOD) δ 4.26, 4.12.

¹H NMR (500 MHz, MeOD) δ 8.20-8.15 (m, 1H, H₈-Naph), 8.00, 7.98 (2s, 1H, H₈), 7.89–7.81 (m, 1H, H₅-Naph), 7.71-7.67 (m, 1H, H₄-Naph), 7.58–7.45 (m, 3H, H₇, H₆, H₂-Naph), 7.43–7.37 (m, 1H, H₄-Naph), 6.00, 5.99 (2s, 1H, H_{1'}), 4.81-4.73 (m, 1H, OCH ester), 4.65–4.55 (m, 2H, H_{5'}), 4.53 (q, *J* = 6.7 Hz, 2H, 6OCH₂CH₃), 4.35-4.22 (m, 2H, H_{3'} and H_{4'}), 4.08-3.99 (m, 1H, CHCH₃), 3.80-3.75 (m, 2H, CH₂ ester), 3.48–3.37 (m, 2H, CH₂ ester), 1.80-1.69 (m, 2H, CH₂ ester), 1.54-1.49 (m, 2H, CH₂ ester), 1.44 (t, *J* = 7.2 Hz, 3H, 6OCH₂CH₃), 1.32, 1.30 (2d, *J* = 6.4 Hz, 3H, CHCH₃), 0.98, 0.97 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.08 (C=O), 162.33 (C6), 161.90 (C2), 154.85 (C4), 148.02, 147.96 (ipso Naph), 139.39, 139.11 (C8), 136.30, 136.26 (C10-Naph), 128.85, 128.80, (Naph), 127.77, 127.73 (C9-Naph), 127.48, 127.45, 127.20, 126.77, 126.55, 126.52, 125.97, 125.90, 123.86, 123.73, 123.59, 123.46, 122.78, 122.73 (Naph), 116.23, 116.11 (2d, ³*J*_{C-C-O-P} = 3.8 Hz, C2-Naph), 115.38 (C5), 93.41, 93.26 (C1'), 83.36, 82.19 (2d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 79.95, 79.91 (C2'), 74.92, 74.71 (OCH ester), 71.30 (C3'), 68.12, 67.67 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 66.06, 66.04 (2x CH₂O ester), 63.65, 63.62 (6OCH₂CH₃), 51.80, 51.74 (CHCH₃), 32.52, 32.48 (2x CH₂ ester), 20.61, 20.43 (2d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.30, 20.26 (2'CCH₃), 14.85 (6OCH₂CH₃).

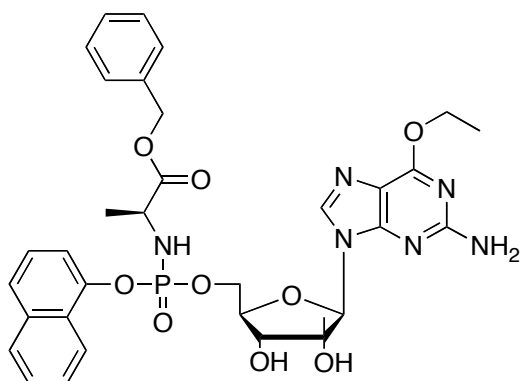
HPLC (System 1) *t*_R = 17.85, 18.24 min.

HPLC (System 2) t_R = 24.23, 24.77 min.

MS (ES+) m/z : 687.25 (MH^+ , 100%).

HRMS calculated for $C_{34}H_{40}N_6O_9P_1$: 687.2571; found 687.2549.

Synthesis of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-alaninyl)] phosphate (5.14c).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.5**, 250 mg, 0.77 mmol), α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3k**, 622 mg, 1.54 mmol), *t*BuMgCl (1.54 ml, 1.54 mmol) in 5 ml of dry THF.

After silica gel column chromatography, using $CHCl_3/MeOH$ (0 to 5%, gradient) as eluent, pure product **5.14c** was obtained as a white foam (68.7 mg of 13%).

^{31}P NMR (202 MHz, MeOD) δ 4.31, 4.26.

1H NMR (500 MHz, MeOD) δ 8.17-8.15 (m, 1H, H_8 -Naph), 7.96, 7.92 (2s, 1H, H_8), 7.85, 7.84 (2d, 1H, J = 8.0 Hz, H_5 -Naph), 7.67, 7.66 (2d, J = 8.0 Hz, 1H, H_4 -Naph), 7.51-7.43 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.36, 7.35 (2t, J = 8.0 Hz, 1H, H_3 -Naph), 7.27-7.22 (m, 5H, OCH_2Ph), 6.00, 5.99 (2s, 1H, H_1'), 5.05- 4.95 (m, 2H, OCH_2Ph), 4.61-4.57 (m, 2H, H_5'), 4.51, 4.50 (2q, J = 7.0 Hz, 2H, $6OCH_2CH_3$) 4.34, 4.27 (2d, J = 9.0 Hz, 1H, H_3'), 4.25-4.22 (m, 1H, H_4'), 4.12-4.05 (m, 1H, $CHCH_3$), 1.42, 1.41 (2t, J = 7.0 Hz, 3H, $6OCH_2CH_3$), 1.30, 1.28 (2d, J = 7.5 Hz, $CHCH_3$), 0.98, 0.95 (2s, 3H, $2'CCH_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.80, 174.61 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.39, 162.38 (C6), 161.85 (C2), 154.58, 154.52 (C4), 147.98 (ipso Naph), 139.26, 138.98 (C8), 137.13 (ipso OCH_2Ph), 136.26, 136.24 (C10-Naph), 129.51, 129.22, 129.15, 129.11, 128.93, 128.79 (Naph, OCH_2Ph), 127.91, 127.88 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.74, 127.72, 127.48, 126.51, 126.48, 125.95, 122.83, 122.75 (Naph, OCH_2Ph), 116.25, 116.23 (C2-Naph), 115.64, 115.62 (C5), 93.32, 93.14 (C1'), 82.29, 82.11 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.96, 79.92 (C2'), 74.94, 74.62 (C3'), 68.02, 67.91 (2d, $^2J_{C-O-P}$ = 3.7 Hz, C5'), 67.49, 67.45 (OCH_2Ph), 63.58, 63.56 ($6OCH_2CH_3$),

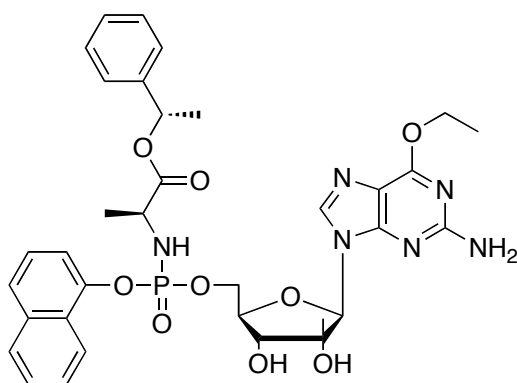
51.81, 51.72 (CHCH₃), 20.59, 20.54, 20.39 (CHCH₃), 20.36, 20.34 (2'CCH₃), 14.89, 14.88 (6OCH₂CH₃).

HPLC (System 1) *t*_R = 22.25, 22.73 min

MS (ES⁺) *m/z*: 693.25 (MH⁺, 100%).

HRMS calculated for C₃₃H₃₈N₆O₉P₁: 693.2438, found 693.2452.

Synthesis of 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(*S*-phenylethoxy-L-alaninyl)] phosphate (5.14d).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-ethyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (**5.5**, 250 mg, 0.77 mmol), α-naphthyl-(*S*-phenylethoxy-L-alaninyl) phosphorochloridate (**3.31**, 678 mg, 1.54 mmol), *t*BuMgCl (1.54 ml, 1.54 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.14d** was obtained as a white foam (120 mg of 22%).

³¹P NMR (202 MHz, MeOD) δ 4.32, 4.29.

¹H NMR (500 MHz, MeOD) δ 8.19- 8.16 (m, 1H, H₈-Naph), 8.05, 8.01 (2s, 1H, H₈), 7.84- 7.78 (m, 1H, H₅-Naph), 7.65, 7.63 (2d, *J* = 8.0 Hz, 1H, H₄-Naph), 7.55–7.45 (m, 3H, H₂, H₇, H₆-Naph), 7.38-7.32 (m, 1H, H₃-Naph), 7.27-7.19 (m, 5H, OCH(CH₃)Ph), 6.02, 6.01 (2s, 1H, H_{1'}), 5.73, 5.69 (2q, *J* = 6.5 Hz, 1H, OCH(CH₃)Ph), 4.67–4.56 (m, 1H, H_{5'}), 4.49 (q, *J* = 7.0 Hz, 2H, 6OCH₂CH₃), 4.32-4.22 (m, 2H, H_{3'} and H_{4'}), 4.10- 4.03 (m, 1H, CHCH₃), 1.41-1.37 (m, 6H, 6OCH₂CH₃ and OCH(CH₃)Ph), 1.28, 1.25 (2d, *J* = 8.0 Hz, 3H, CHCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.26, 174.00 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 162.24, 162.21 (C6), 161.82, 161.75 (C2), 154.25 (C4), 147.99, 147.97, 147.92 (ipso Naph), 142.84, 142.80 (ipso OCH(CH₃)Ph), 139.19, 138.88 (C8), 136.23, 136.21 (2d, ³*J*_{C-C-O-P} = 3.7 Hz, C10-Naph), 129.74, 129.55, 129.51, 128.97, 128.88, 128.84, 128.63 (Naph and OCH(CH₃)Ph), 127.87, 127.81 (2d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.76,

127.50, 127.31, 127.15, 127.02, 126.98, 126.79, 126.72, 126.55, 126.52, 126.38, 126.01, 125.99, 124.22, 123.49, 122.81, 122.75 (Naph and OCH(CH₃)Ph), 116.30, 116.24 (2d, ³J_{C-C-O-P} = 3.7 Hz, C2-Naph), 115.06, 114.94 (C5), 93.38, 93.22 (C1'), 82.39, 82.24 (2d, ³J_{C-C-O-P} = 8.8 Hz, C4'), 80.00, 79.94 (C2'), 74.91, 74.86, 74.77, 74.64, 74.50 (C3' and OCH(CH₃)Ph), 68.01, 67.53 (2d, ²J_{C-O-P} = 5.0 Hz, C5'), 63.86, 63.81 (6OCH₂CH₃), 51.91, 51.82 (CHCH₃), 22.66, 22.60 (OCH(CH₃)Ph), 20.60, 20.55 (CHCH₃), 20.41, 20.35 (2'CCH₃), 14.92 (6OCH₂CH₃).

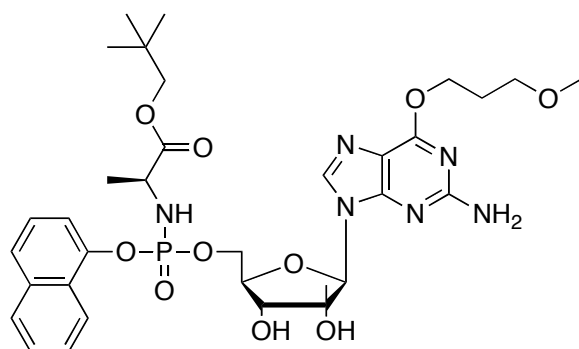
HPLC (System 1) t_R = 22.41, 22.93 min

HPLC (System 2) t_R = 28.28, 28.89 min

MS (ES+) m/z: 707.26 (MH⁺, 100%).

HRMS calculated for C₃₄H₄₀N₆O₉P₁: 707.2594, found 707.2560.

Synthesis of 2-amino-6-*O*-(3-methoxypropyl)-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.17a).



Prepared according to the Standard Procedure 7 from: 2-amino-6-*O*-(3-methoxypropyl)-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (**5.8**, 40 mg, 0.11 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)

phosphorochloridate (**3.3e**, 83 mg, 0.22 mmol), *t*BuMgCl (0.22 ml, 0.22 mmol) in 2 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.14d** was obtained as a white foam (19 mg of 25%).

³¹P NMR (202 MHz, MeOD) δ 4.28, 4.21.

¹H NMR (500 MHz, MeOD) δ 8.19–8.15 (m, 1H, H₈-naph), 7.98, 7.95 (2s, 1H, H₈), 7.89–7.85 (m, 1H, H₈ and H₅-naph), 7.70, 7.68 (2d, *J* = 8.5 Hz, 1H, H₄-naph), 7.54–7.45 (m, 3H, H₇, H₆, H₂-naph), 7.42–7.37 (m, 1H, H₃-naph), 6.00, 5.98 (2s, 1H, H₁'), 4.65–4.57 (m, 2H, H₅'), 4.55 (t, *J* = 6.3 Hz, 2H, 6OCH₂CH₂CH₂OCH₃), 4.35–4.22 (m, 2H, H₃' and H₄'), 4.11–4.03 (m, 1H, CHCH₃), 3.78–3.58 (m, 4H, OCH₂C(CH₃)₃ and 6OCH₂CH₂CH₂OCH₃), 3.37, 3.36 (2s, 3H, 6OCH₂CH₂CH₂OCH₃), 2.09–2.06 (m, 2H,

6OCH₂CH₂CH₂OCH₃), 1.33, 1.32 (2d, J = 7.0 Hz, 3H, CHCH₃), 0.98, 0.97 (2s, 3H, 2'CCH₃), 0.87, 0.86 (2s, 9H, OCH₂C(CH₃)₃).

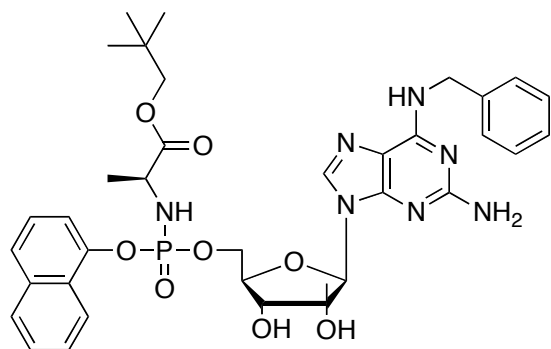
¹³C NMR (126 MHz, MeOD) δ 174.80, 174.76 (C=O), 162.72, 162.37 (C6), 161.84 (C2), 156.64, 154.60 (C4), 147.98 (ipso Naph), 139.40, 139.12 (C8), 136.30, 136.28 (C10-Naph), 128.82, 128.77 (CH-Naph), 127.89, 127.71 (2d, d, $^3J_{C-C-O-P}$ = 5.0 Hz, C9-Naph), 127.52, 127.50, 126.53, 126.49, 125.98, 122.82, 122.75 (CH-Naph), 116.21, 116.17 (C2-naph), 115.56 (C5), 93.37, 93.22 (C1'), 82.33, 82.17 (d, $^3J_{C-C-O-P}$ = 7.5 Hz, C4'), 79.96, 79.90 (C2'), 75.34 (OCH₂C(CH₃)₃), 74.95, 74.71 (C3'), 70.32 (6OCH₂CH₂CH₂OCH₃), 67.83, 67.27 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 64.72 (6OCH₂CH₂CH₂OCH₃), 58.92 (6OCH₂CH₂CH₂OCH₃), 51.79, 51.73 (CHCH₃), 32.24, 32.21 (OCH₂C(CH₃)₃), 30.24 (6OCH₂CH₂CH₂OCH₃), 26.68, 26.67 (OCH₂C(CH₃)₃), 20.79, 20.58 (2d, $^3J_{C-C-N-P}$ = 6.20 Hz, CHCH₃), 20.29, 20.27 (2'CCH₃).

HPLC t_R = 19.15, 19.48 min.

MS (ES+) m/z : 717.30 (MH⁺, 100%).

HRMS calculated for C₃₃H₄₆N₆O₁₀P₁: 717.3013, found 717.3004.

Synthesis of 2-amino-6-NH-benzyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.18a).



Prepared according to the Standard Procedure 7 from: 2-amino-6-NH-benzyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine (**5.9**, 150 mg, 0.39 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)

phosphorochloridate (**3.3e**, 303 mg, 0.79 mmol), *t*BuMgCl (0.79 ml, 0.79 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.18a** was obtained as a white foam (33 mg of 11%).

³¹P NMR (202 MHz, MeOD) δ 4.43, 4.23.

¹H NMR (500 MHz, MeOD) δ 8.19–8.17 (m, 1H, H₈-naph), 7.87 (s, 1H, H₈), 7.86–7.81 (m, 1H, H₅-Naph) 7.70–7.65 (m, 1H, H₄-Naph), 7.54–7.50 (m, 3H, H₂, H₇, H₆-

Naph), 7.46- 7.36 (m, 3H, H₃-Naph and 6NHCH₂Ph), 7.32- 7.29 (m, 2H, 6NHCH₂Ph), 7.25- 7.22 (m, 1H, 6NHCH₂Ph), 5.98, 5.97 (2s, 1H, H₁'), 4.75- 4.72 (m, 2H, 6NHCH₂Ph), 4.68- 4.57 (m, 2H, H₅'), 4.30- 4.23 (m, 2H, H₃' and H₃'), 4.10-4.03 (m, 1H, CHCH₃), 3.76, 3.71, 3.63, 3.59 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.32, 1.30 (2d, J = 7.4 Hz, 1H, CHCH₃), 0.99, 0.98 (2s, 3H, 2'CCH₃), 0.85, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.06 (d, $^3J_{C-C-N-P}$ = 4.7, C=O), 174.82 (d, $^3J_{C-C-N-P}$ = 5.8, C=O), 162.15 (C6), 156.46 (C4), 148.02, 147.96 (d, $^2J_{C-O-P}$ = 2.0, ipso Naph), 140.61 (ipso 6NHCH₂Ph), 137.17, 136.87 (C8), 136.30 (C10-Naph), 129.53, 129.52, 128.86, 128.80, 128.65, 128.64, 128.15 (CH-Naph and 6NHCH₂Ph), 127.91, 127.86 (2d, $^3J_{C-C-O-P}$ = 2.5, C9-Naph), 127.77, 127.75 (CH-Naph), 127.50, 127.49, 126.53, 126.47, 125.97, 122.81, 122.74 (CH-Naph, 6NHCH₂Ph), 116.20 (d, $^3J_{C-C-O-P}$ = 3.3 Hz, C2-Naph), 116.17 (d, $^3J_{C-C-O-P}$ = 4.5 Hz, C2-Naph), 114.78, 114.75 (C5), 92.98, 92.79 (C1'), 82.13, 81.98 (2d, $^3J_{C-C-O-P}$ = 8.3 Hz, C4'), 79.98, 79.90 (C2'), 75.37, 75.35 (OCH₂C(CH₃)₃), 74.82, 74.51 (C3'), 67.87, 67.29 (2d, $^2J_{C-O-P}$ = 5.2 Hz, C5'), 51.78 (CHCH₃), 44.91 (6NHCH₂Ph), 32.24, 32.21 (OCH₂C(CH₃)₃), 26.70, 26.69 (OCH₂C(CH₃)₃), 20.84, 20.62 (2d, $^3J_{C-C-N-P}$ = 6.2 Hz, CHCH₃), 20.38, 20.35 (2'CCH₃).

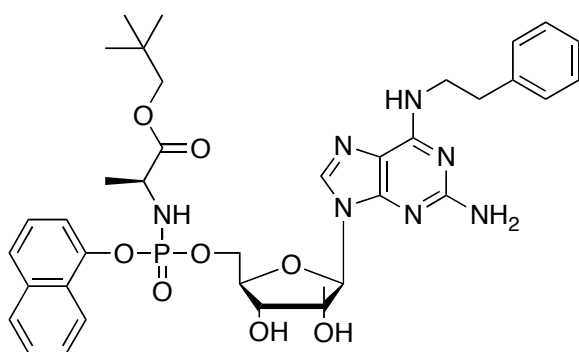
HPLC t_R = 22.41, 22.93 min

MS (ES+) m/z : 733.30 (MH⁺, 100%).

HRMS calculated for C₃₆H₄₅N₇O₈P₁: 733.3057, found 733.3041.

CHN calculated for C₃₆H₄₅N₇O₈P₁ + 0.75 H₂O: C 57.86, H 6.14, N 13.12; found: C 57.90, H 5.98, N 13.01.

Synthesis of 2-amino-6-NH-phenylethyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (5.19a).



Prepared according to the Standard Procedure 7 from: 2-amino-6-NH-phenylethyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine (**5.10**, 200 mg, 0.50 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)

phosphorochloridate (**3.3e**, 367 mg, 1.01 mmol), *t*BuMgCl (1.01 ml, 1.01 mmol) in 5 ml of dry THF. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent, pure product **5.18a** was obtained as a white foam (247 mg of 24%).

³¹P NMR (202 MHz, MeOD) δ 4.25.

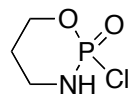
¹H NMR (500 MHz, MeOD) δ 8.19, 8.16 (2d, *J* = 8.5 Hz, 1H, H₈-naph), 7.87–7.81 (m, 2H, H₈ and H₅-naph), 7.69, 7.67 (2d, *J* = 8.5 Hz, 1H, H₄-naph), 7.54–7.47 (m, 3H, H₇, H₆, H₂-naph), 7.42–7.37 (m, 1H, H₃-naph), 7.27–7.16 (m, 5H, NHCH₂CH₂Ph), 5.98, 5.97 (2s, 1H, H₁'), 4.69–4.58 (m, 2H, H₅'), 4.27–4.22 (m, 2H, H₃' and H₄'), 4.11–4.03 (m, 1H, CHCH₃), 3.78–3.58 (m, 4H, OCH₂C(CH₃)₃ and NHCH₂CH₂Ph), 2.94 (t, *J* = 7.7 Hz, 2H, NHCH₂CH₂Ph), 1.33, 1.32 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 0.98, 0.96 (2s, 3H, 2'CCH₃), 0.86, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.06 (d, ³*J*_{C-C-N-P} = 4.7, C=O), 174.83 (d, ³*J*_{C-C-N-P} = 5.8, C=O), 162.12 (C6), 156.51 (C4), 148.01 (ipso Naph), 140.69 (ipso NHCH₂CH₂Ph), 137.04, 136.76 (C8), 136.30, 136.28 (C10-Naph), 129.91, 129.50, 128.87, 128.82 (CH-Naph and NHCH₂CH₂Ph), 127.93, 127.76 (C9-Naph), 127.52, 127.50, 127.31, 126.53, 126.49, 125.98, 122.82, 122.75 (CH-Naph and NHCH₂CH₂Ph), 116.22, 116.20 (C2-naph), 114.81 (C5), 92.95, 92.77 (C1'), 82.11 (d, ³*J*_{C-C-O-P} = 7.7 Hz, C4'), 81.97 (d, ³*J*_{C-C-O-P} = 8.5, C4'), 79.98, 79.91 (C2'), 75.38, 75.36 (OCH₂C(CH₃)₃), 74.80, 74.49 (C3'), 67.83, 67.27 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 51.79, 51.73 (CHCH₃), 44.52 (NHCH₂CH₂Ph), 36.82 (OCH₂C(CH₃)₃), 32.26, 32.23 (NHCH₂CH₂Ph), 26.72, 26.71 (OCH₂C(CH₃)₃), 20.87 (d, ³*J*_{C-C-N-P} = 6.20 Hz, CHCH₃), 20.64 (d, ³*J*_{C-C-N-P} = 7.70 Hz, CHCH₃), 20.39, 20.37 (2'CCH₃).

HPLC *t*_R = 22.75, 22.93 min.

MS (ES+) *m/z*: 748.34 (MH⁺, 100%).

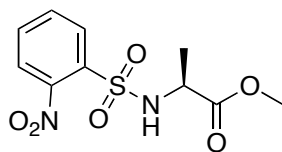
HRMS calculated for C₃₇H₄₆N₇O₉P₁: 770.3043, found 770.3079.

12.6 Experimental section – Chapter Seven.**Synthesis of 2-chloro-1,3,2-oxazaphosphinane-2-oxide (7.1).**

POCl_3 (4.58 ml, 49.14 mmol) in 40 ml of dry CHCl_3 was cooled down to $-15\text{ }^\circ\text{C}$. A solution of 3-aminopropan-1-ol (3.79 ml, 49.14 mmol) in dry CHCl_3 (10 ml), containing Et_3N (7.00 ml, 50.00 mmol) was added dropwise with stirring to the mixture which was kept at $-10\text{ }^\circ\text{C}$, followed by the addition of further quantity (7 ml, 50.00 mmol) of Et_3N in dry CHCl_3 (10 ml). The reaction mixture was stirred at $0\text{ }^\circ\text{C}$ for 18 h. After that time, solvent was removed under reduced pressure at $35\text{ }^\circ\text{C}$. The solid residue was extracted with dry acetone (4x 20 ml), and evaporated to dryness. The crude mixture was purified on silica gel (flash chromatography), using EtOAc/Hexane (1:1) as eluent. Desired compound **7.1** was obtained as a white solid (6.50 g, 84%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.22.

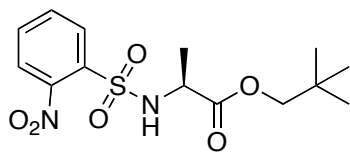
^1H NMR (500 MHz, CDCl_3) δ 4.57- 4.47 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 4.07 (s, 1H, NH), 3.46- 3.36 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.25- 2.16, 1.80- 1.76 (2m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$).

Synthesis of *N*-(2-nitrophenylsulfonyl)-L-alanine methyl ester (7.3a).

Prepared according to the Standard Procedure 9, starting from: L-alanine methyl ester hydrochloride (5.00 g, 35.82 mmol), Et_3N (11.47 ml, 82.38 mmol) and 2-nitrobenzenesulfonyl chloride (7.94 g, 35.82 mmol) in dry DCM (250 ml). After work up, the pure compound **7.3a** was obtained as a yellow oil (10.36 g, 100%).

^1H NMR (500 MHz, MeOD) δ 8.09-8.07 (m, 1H, $\text{H}_3\text{-Ar}$), 7.92- 7.90 (m, 1H, $\text{H}_6\text{ Ar}$), 7.77- 7.73 (m, 1H, H_4 and $\text{H}_5\text{ Ar}$), 5.99 (bs, 1H, NH), 4.24 (q, $J= 7.00\text{ Hz}$, 1H, CHCH_3), 3.52 (s, 3H, OCH_3), 1.47 (d, $J= 7.00\text{ Hz}$, 3H, CHCH_3).

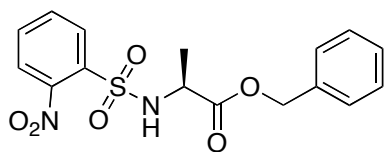
Synthesis of *N*-(2-nitrophenylsulphonyl)-L-alanine 2,2-dimethylpropyl ester (**7.3b**).



Prepared according to the Standard Procedure 9, starting from: L-alanine 2,2-dimethylpropyl ester hydrochloride (8.00 g, 15.09 mmol), Et₃N (4.83 ml, 34.70 mmol) and 2-nitrobenzenesulfonyl chloride (3.34 g, 15.09 mmol) in dry DCM (400 ml). After work up, the pure compound **7.3b** was obtained as a yellow oil (7.64 g, 92%).

¹H NMR (500 MHz, CDCl₃) δ 8.11- 8.09 (m, 1H, H₃-Ar), 7.94- 7.92 (m, 1H, H₄-Ar), 7.76- 7.72 (m, 2H, H₅-Ar and H₄-Ar), 4.29 (q, *J*= 7.5 Hz, 1H, CHCH₃), 3.68, 3.65 (AB, *J*_{AB}= 12.00 Hz, 2H, OCH₂C(CH₃)₃), 1.53 (d, *J*= 7.5 Hz, 3H, CHCH₃), 0.87 (s, 9H, OCH₂C(CH₃)₃).

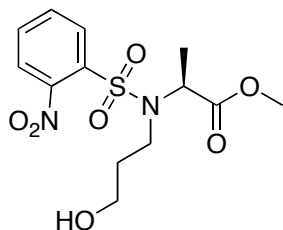
Synthesis of *N*-(2-nitrophenylsulphonyl)-L-alanine benzyl ester (**7.3c**).



Prepared according to the Standard Procedure 9, starting from: L-alanine benzyl ester hydrochloride (5.00 g, 14.23 mmol), Et₃N (4.55 ml, 32.72 mmol) and 2-nitrobenzenesulfonyl chloride (3.15 g, 14.23 mmol) in dry DCM (250 ml). After work up, the pure compound **7.3c** was obtained as a yellow oil (5.80 g, 100%).

¹H NMR (500 MHz, CDCl₃) δ 8.03 (d, *J*= 8.0 Hz, 1H, H₃-Ar), 7.84 (d, *J*= 6.5 Hz, 1H, H₄-Ar), 7.69-7.62 (m, 2H, H₅-Ar and H₄-Ar), 7.36-7.20 (m, 5H, OCH₂Ph), 4.98, 4.95 (AB, *J*_{AB}= 8.5 Hz, 2H, OCH₂Ph), 4.33 (q, *J*= 7.0 Hz, 1H, CHCH₃), 1.52 (d, *J*= 7.0 Hz, 3H, CHCH₃).

Synthesis of *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine methyl ester (**7.3a'**).

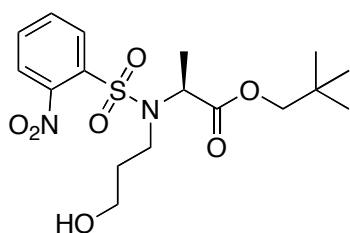


Prepared according to the Standard Procedure 10, starting from: *N*-(2-nitrophenylsulphonyl)-L-alanine methyl ester (**7.3a**, 10.36 g, 35.94 mmol), cesium carbonate (11.71 g,

35.94 mmol) and 3-bromopropanol (4.71 ml, 53.91 mmol) in dry DMF (160 ml). After work up and column chromatography using EtOAc/petroleum ether (8:2) as an eluent, the pure compound **7.3a'** was obtained as a yellow oil (4.15 g, 31%).

^1H NMR (500 MHz, MeOD) δ 8.12 (d, J = 9.5 Hz, 1H, $\text{H}_3\text{-Ar}$), 7.84-7.79 (m, 2H, H_5 and H_6 Ar), 7.75- 7.74 (m, 1H, H_4 Ar), 4.76 (q, J = 7.5 Hz, 1H, CHCH_3), 3.61- 3.54 (m, 6H, OCH_3 , $\text{NCH}_{2a}\text{CH}_2\text{CH}_2\text{OH}$ and $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.35-3.28 (m, 1H, $\text{NCH}_{2b}\text{CH}_2\text{CH}_2\text{OH}$), 1.96- 1.82 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.53 (d, J = 7.5 Hz, 3H, CHCH_3).

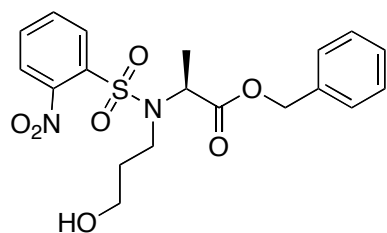
Synthesis of *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine 2,2-dimethylpropyl ester (7.3b'**).**



Prepared according to the Standard Procedure 10, starting from: *N*-(2-nitrophenylsulphonyl)-L-alanine 2,2-dimethylpropyl ester (**7.3b**, 7.64 g, 22.18 mmol), cesium carbonate (7.23 g, 22.18 mmol) and 3-bromopropanol (2.90 ml, 33.28 mmol) in dry DMF (150 ml). After work up and column chromatography using EtOAc/petroleum ether (8:2) as an eluent, the pure compound **7.3b'** was obtained as a yellow oil (2.17 g, 29%).

^1H NMR (500 MHz, CDCl_3) δ 8.06 (d, J = 7.0 Hz, 1H, $\text{H}_3\text{-Ar}$), 7.70-7.67 (m, 2H, H_6 and H_5 Ar), 7.60-7.59 (m, 1H, $\text{H}_4\text{-Ar}$), 4.81 (q, J = 7.5 Hz, 1H, CHCH_3), 3.78-3.64 (m, 4H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$ and $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.60-3.55 (m, 1H, $\text{NCH}_{2a}\text{CH}_2\text{CH}_2\text{OH}$), 3.93-3.33 (m, 1H, 1 of $\text{NCH}_{2b}\text{CH}_2\text{CH}_2\text{OH}$), 1.90-1.83 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.55 (d, J = 7.5 Hz, 3H, CHCH_3), 0.89 (s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

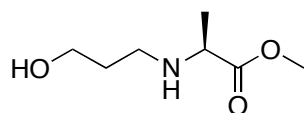
Synthesis of *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine 2,2-dimethylpropyl ester (7.3c'**).**



Prepared according to the Standard Procedure 10, starting from: *N*-(2-nitrophenylsulphonyl)-L-alanine benzyl ester (**7.3c**, 5.80 g, 18.16 mmol), cesium carbonate (5.92 g, 18.16 mmol) 3-bromopropanol (2.38 ml, 27.24 mmol) and in dry DMF (80 ml). After work up and column chromatography using EtOAc/petroleum ether (8:2) as an eluent, the pure compound **7.3c'** was obtained as a yellow oil (1.92 g, 25%).

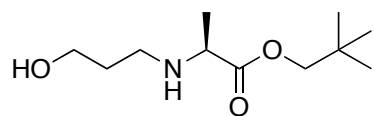
^1H NMR (500 MHz, MeOD) δ 8.05 (d, J = 8.0 Hz, 1H, $\text{H}_3\text{-Ar}$), 7.72-7.62 (m, 3H, H_4 , H_5 and H_6 Ar), 7.33-7.25 (m, 5H, OCH_2Ph), 5.05, 5.00 (AB, J_{AB} = 11.50 Hz, 2H, OCH_2Ph), 4.11 (q, J = 7.5 Hz, 1H, CHCH_3), 3.61-3.53 (m, 3H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$ and $\text{NCH}_{2\text{a}}\text{CH}_2\text{CH}_2\text{OH}$), 3.32-3.27 (m, 1H, $\text{NCH}_{2\text{b}}\text{CH}_2\text{CH}_2\text{OH}$), 1.94- 1.83 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.55 (d, J = 7.5 Hz, 3H, CHCH_3).

Synthesis of *N*-(3-hydroxypropyl)- L-alanine methyl ester (7.3a''**).**



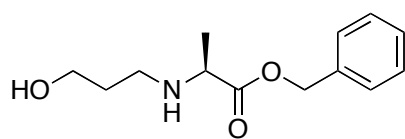
Prepared according to the Standard Procedure 11, starting from: *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine methyl ester (**7.3a'**, 3.85 g, 11.12 mmol), potassium carbonate (4.98 g, 36.13 mmol) and thiophenol (1.25 ml, 27.24 mmol) in dry ACN (60 ml). After work up and column chromatography using $\text{CHCl}_3/\text{MeOH}$ (9:1) as an eluent, the pure compound **7.3a''** was obtained as a yellowish oil (0.76 g, 42%).

^1H NMR (500 MHz, CDCl_3) δ 3.74 (t, J = 5.5 Hz, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 3.70 (s, 3H, OCH_3), 3.35 (q, J = 7.0 Hz, 1H, CHCH_3), 3.02 (bs, 1H, NH), 2.88-2.83 (m, 1H, $\text{NHCH}_{2\text{a}}\text{CH}_2\text{CH}_2\text{OH}$), 2.65-2.60 (m, 1H, $\text{NHCH}_{2\text{b}}\text{CH}_2\text{CH}_2\text{OH}$), 1.68-1.64 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{OH}$), 1.26 (d, J = 7.0 Hz, 3H, CHCH_3).

Synthesis of *N*-(3-hydroxypropyl)-L-alanine 2,2-dimethylpropyl ester (7.3b'').

Prepared according to the Standard Procedure 11, starting from: *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine 2,2-dimethylpropyl ester (**7.3b'**, 2.17 g, 6.30 mmol), potassium carbonate (2.83 g, 20.51 mmol) and thiophenol (0.71 ml, 6.93 mmol) in dry ACN (60 ml). After work up and column chromatography using CHCl₃/MeOH (9:1) as an eluent, the pure compound **7.3b''** was obtained as a yellowish oil (0.91 g, 66%).

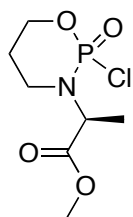
¹H NMR (500 MHz, CDCl₃) δ 3.80, 3.78 (AB, *J*_{AB}=10.5 Hz, 2H, OCH₂C(CH₃)₃), 3.73 (t, *J*= 5.5 Hz, 2H, NHCH₂CH₂CH₂OH), 3.35 (q, *J*=7.0 Hz, 1H, CHCH₃), 3.00 (bs, 1H, NH), 2.89-2.83, 2.65-2.60 (2m, 2H, NHCH₂CH₂CH₂OH), 1.68-1.63 (m, 2H, NHCH₂CH₂CH₂OH), 1.28 (d, *J*=7.0 Hz, 3H, CHCH₃), 0.91 (s, 9H, OCH₂C(CH₃)₃).

Synthesis of *N*-(3-hydroxypropyl)-L-alanine benzyl ester (7.3c'').

Prepared according to the Standard Procedure 11, starting from: *N*-(3-hydroxypropyl)-*N*-(2-nitrophenylsulphonyl)-L-alanine benzyl ester (**7.3c'**, 1.92 g, 4.54 mmol), potassium carbonate (2.04 g, 14.77 mmol) and thiophenol (0.51 ml, 5.00 mmol) in dry ACN (60 ml). After work up and column chromatography using CHCl₃/MeOH (9:1) as an eluent, the pure compound **7.3c''** was obtained as a yellowish oil (0.72 g, 67%).

¹H NMR (500 MHz, CDCl₃) δ 7.39-7.32 (m, 5H, OCH₂Ph), 5.19, 5.15 (AB, *J*_{AB}=12.5 Hz, 2H, OCH₂Ph), 3.77 (t, *J*= 5.5 Hz, 2H, NHCH₂CH₂CH₂OH), 3.42 (q, *J*=7.0 Hz, 1H, CHCH₃), 2.95 (bs, 1H, NH), 2.91-2.87 (m, 1H, NHCH_{2a}CH₂CH₂OH), 2.68-2.64 (m, 1H, NHCH_{2b}CH₂CH₂OH), 1.71-1.66 (m, 2H, NHCH₂CH₂CH₂OH), 1.32 (d, *J*=7.0 Hz, 3H, CHCH₃).

Synthesis of (2S)-methyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (7.2a).

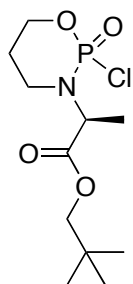


Prepared according to the Standard Procedure 12, starting from: *N*-(3-hydroxypropyl)- L-alanine methyl ester (**7.3a''**, 0.40 g, 2.48 mmol) and Et₃N (0.35 ml, 2.48 mmol) in dry CHCl₃ (2.5 ml). POCl₃ (0.23 ml, 2.48 mmol) was dissolved in dry CHCl₃ (0.5 ml) and the reaction mixture was cooled down to -15 °C. A solution of *N*-(3-hydroxypropyl)- L-alanine methyl ester was added dropwise to the POCl₃ solution, followed by the addition of further quantity of Et₃N (0.35 ml, 2.48 mmol) in dry CHCl₃ (0.5 ml). The reaction mixture was stirred at 0 °C for 18 h. After purification on silica gel (flash chromatography), using EtOAc/Hexane (1:1) as eluent, the compound **7.2a** was obtained as a clear, colorless oil (0.52 g, 87%).

³¹P NMR (202 MHz, CDCl₃) δ 9.27, 8.62.

¹H NMR (500 MHz, CDCl₃) δ 4.69-4.57 (m, 1H, CHCH₃), 4.55- 4.38 (m, 2H, NCH₂CH₂CH₂O), 3.73 (s, 3H, OCH₃), 3.47-3.16 (m, 2H, NCH₂CH₂CH₂O), 2.42-2.40, 2.21- 2.07 (2m, 1H, NCH₂CH_{2a}CH₂O), 1.91-1.80 (m, 1H, NCH₂CH_{2b}CH₂O), 1.46, 1.45 (2d, *J* = 7.0 Hz, 3H, CHCH₃).

(2S)-neopentyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl) propanoate (7.2b)

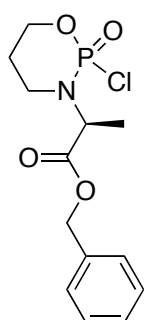


Prepared according to the Standard Procedure 12, starting from: *N*-(3-hydroxypropyl)- L-alanine 2,2-dimethylpropyl ester (**7.3b''**, 0.40 g, 1.84 mmol) and Et₃N (0.25 ml, 1.84 mmol) in dry CHCl₃ (1.84 ml). POCl₃ (0.17 ml, 1.84 mmol) was dissolved in dry CHCl₃ (0.37 ml) and the reaction mixture was cooled down to -15 °C. A solution of *N*-(3-hydroxypropyl)- L-alanine 2,2-dimethylpropyl ester was added dropwise to the POCl₃ solution, followed by the addition of further quantity of Et₃N (0.25 ml, 1.84 mmol) in dry CHCl₃ (0.37 ml). The reaction mixture was stirred at 0 °C for 18 h. After purification on silica gel (flash chromatography), using EtOAc/Hexane (1:1) as eluent, the compound **7.2b** was obtained as a clear, colorless oil (0.41 g, 75%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.04, 8.62.

^1H NMR (500 MHz, CDCl_3) δ 4.71-4.61 (m, 1H, CHCH_3), 4.55-4.42 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.88-3.79 (m, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 3.49-3.28 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.41-2.34, 2.18-2.09, 1.89-1.83 (3m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.47, 1.45 (2d, $J=7.5$ Hz, 3H, CHCH_3), 0.97, 0.96 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of (2S)-benzyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl) propanoate (7.2c).

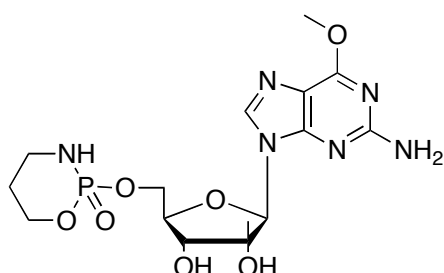


Prepared according to the Standard Procedure 12, starting from: *N*-(3-hydroxypropyl)-L-alanine benzyl ester (**7.3c''**, 0.35 g, 1.47 mmol) and Et_3N (0.21 ml, 1.47 mmol) in dry CHCl_3 (1.47 ml). POCl_3 (0.14 ml, 1.47 mmol) was dissolved in dry CHCl_3 (0.30 ml) and the reaction mixture was cooled down to -15°C . A solution of *N*-(3-hydroxypropyl)-L-alanine benzyl ester was added dropwise to the POCl_3 solution, followed by the addition of further quantity of Et_3N (0.21 ml, 1.47 mmol) in dry CHCl_3 (0.30 ml). The reaction mixture was stirred at 0°C for 18 h. After purification on silica gel (flash chromatography), using EtOAc/Hexane (1:1) as eluent, the compound **7.2c** was obtained as a clear, colorless oil (0.42 g, 90%).

^{31}P NMR (202 MHz, CDCl_3) δ 9.15, 8.61

^1H NMR (500 MHz, CDCl_3) δ 7.37-7.32 (m, 5H, OCH_2Ph), 5.20-5.11 (m, 2H, OCH_2Ph), 4.69-4.60 (m, 1H, CHCH_3), 4.48-4.37 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 3.34-3.14 (m, 2H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 2.25-2.18, 2.12-2.07, 1.84-1.74 (3m, 1H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{O}$), 1.44, 1.42 (2d, $J=7.5$ Hz, 3H, CHCH_3).

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-1'',3'',2''-oxazaphosphacyclohex-2''-oxide (7.4a).



Prepared according to the Standard Procedure 8, starting from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 400 mg, 1.28 mmol), 2-chloro-1,3,2-oxazaphosphinan-2-

oxide (**7.1**, 399 mg, 2.57 mmol), in 10 ml of dry pyridine. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{EtOH}$ (0 to 5%, gradient), to give a pure product as white foam (**7.4a**, 87 mg, 16%).

^{31}P NMR (202 MHz, MeOD) δ 5.50, 5.46

^1H NMR (500 MHz, MeOD) δ 8.06, 8.05 (2s, 1, H_8), 6.04, 6.03 (2s, 1H, $\text{H}_{1'}$), 4.45-4.31 (m, 4H, $\text{H}_{5'}$ and $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 4.29- 4.22 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{4'}$), 4.07 (s, 3H, 6OCH_3), 3.29- 3.23 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.01- 1.4 (m, 1H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.70- 1.67 (m, 1H, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.01, 1.00 (2s, 3H, $2'\text{CCH}_3$).

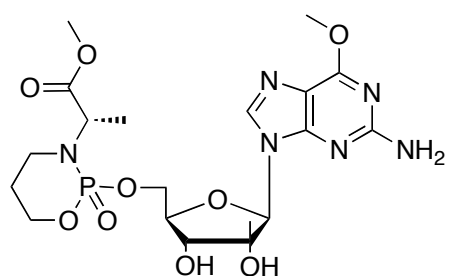
^{13}C NMR (126 MHz, MeOD) δ 162.73 (C6), 161.97 (C2), 154.66 (C4), 138.80, 138.75 (C8), 115.37 (C5), 92.84, 92.79 ($\text{C}_{1'}$), 82.15, 82.09 ($\text{C}_{4'}$), 80.16, 80.14 ($\text{C}_{2'}$), 74.28, 74.20 ($\text{C}_{3'}$), 71.38, 71.31 (2d, $^2J_{\text{C-O-P}} = 6.3$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 65.64, 65.60 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, $\text{C}_{5'}$), 54.29 (6OCH_3), 42.30 (d, $^2J_{\text{C-N-P}} = 11.3$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 42.24 (d, $^2J_{\text{C-N-P}} = 2.50$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 27.38, 27.32 (2d, $^2J_{\text{C-C-O-P}} = 7.55$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 20.28 ($2'\text{CCH}_3$).

HPLC (System 1) $t_R = 5.72, 6.04$ min.

MS (EI+) m/z : 431.13 (MH^+ , 100%).

HRMS calculated for $\text{C}_{15}\text{H}_{29}\text{N}_6\text{O}_7\text{P}_1$: 430.1366, found 430.1361.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-(2*S*)-methyl 2-(2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (7.4b**).**



Prepared according to the Standard Procedure 8, starting from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), (2*S*)-methyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (**7.2a**,

310 mg, 0.96 mmol), in 7 ml of dry pyridine. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (0 to 5%, gradient), to give a pure product as white foam (**7.4b**, 55 mg, 11%).

^{31}P NMR (202 MHz, MeOD) δ 4.62, 4.48.

^1H NMR (500 MHz, MeOD) δ 8.02 (s, 1H, H₈), 6.01, 6.00 (2s, 3H, H_{1'}), 4.40- 4.22 (m, 7H, H_{3'}, H_{4'}, H_{5'}, CHCH₃ and OCH₂CH₂CH₂N), 4.07 (s, 3H, 6OCH₃), 3.74, 3.73 (2s, 3H, OCH₃), 3.30-3.21 (m, 2H, OCH₂CH₂CH₂N), 2.24-2.15 (m, 1H, OCH₂CH_{2a}CH₂N), 1.84-1.81 (m, 1H, OCH₂CH₂CH₂N), 1.42 (d, J = 7.5 Hz, CHCH₃), 1.01 (s, 3H, 2'CCH₃).

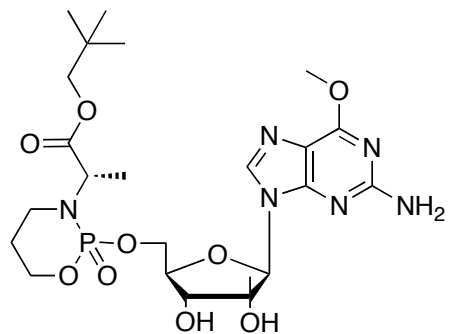
^{13}C NMR (126 MHz, MeOD) δ 174, 23, 174.18 (C=O), 162.76 (C6), 161.99 (C2), 154.60 (C4), 138.93 (C8), 115.51 (C5), 93.04 (C1'), 82.17, 82.10 (C4'), 80.05 (C2'), 71.08, 71.03 (OCH₂CH₂CH₂N), 66.33, 66.29 (C5'), 54.23 (6OCH₃), 52.79 (CHCH₃), 52.79, 52.73 (OCH₃ ester), 44.22, 43.80 (OCH₂CH₂CH₂N), 27.12, 27.07 (OCH₂CH₂CH₂N), 20.24 (2'CCH₃), 16.07, 16.05 (CHCH₃).

HPLC (System 1) t_R = 8.17 min.

MS (EI+) m/z : 516.71 (MH⁺, 100%).

HRMS calculated for C₁₉H₂₉N₆O₇P₁: 516.1734, found 516.1739.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-(2*S*)-neopentyl 2-(2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (7.4c).



Prepared according to the Standard Procedure 8, starting from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), (2*S*)-neopentyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (**7.2b**, 410 mg, 0.96 mmol), in 7 ml of dry pyridine. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product as white foam (**7.4c**, 15.5 mg, 3%).

^{31}P NMR (202 MHz, MeOD) δ 4.46.

^1H NMR (500 MHz, MeOD) δ 8.03 (s, 1H, H₈), 6.01 (s, 1H, H_{1'}), 4.45-4.30 (m, 6H, H_{3'}, H_{5'}, CHCH₃ and OCH₂CH₂CH₂N), 4.24-4.22 (m, 1H, H_{4'}), 4.08 (s, 3H, 6OCH₃), 3.90, 3.81 (AB, J_{AB} =10.5 Hz, 2H, OCH₂C(CH₃)₃), 3.31-3.23 (m, 2H,

OCH₂CH₂CH₂N), 2.24-2.15, 1.85-1.82 (2m, 2H, OCH₂CH₂CH₂N), 1.44 (d, $J=7.5$ Hz, 3H, CHCH₃), 1.01 (s, 3H, 2'CCH₃), 0.98 (s, 9H, OCH₂C(CH₃)₃).

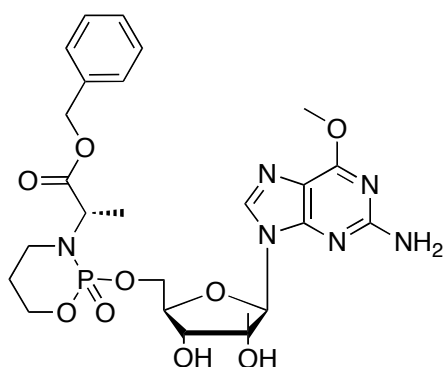
¹³C NMR (126 MHz, MeOD) δ 173.73 (C=O), 162.76 (C6), 162.01 (C2), 154.55 (C4), 138.94 (C8), 115.52 (C5), 93.06 (C1'), 82.16 (d, $^3J_{C-C-O-P} = 8.8$ Hz, C4'), 80.04 (C2'), 75.51 (OCH₂C(CH₃)₃), 74.43 (C3'), 70.99 (d, $^2J_{C-O-P} = 7.6$ Hz, OCH₂CH₂CH₂N), 66.36 (d, $^2J_{C-N-P} = 6.3$ Hz, C5'), 55.33 (d, $^2J_{C-N-P} = 5.5$ Hz, CHCH₃), 54.20 (6OCH₃), 44.08 (OCH₂CH₂CH₂N), 32.29 (OCH₂C(CH₃)₃), 27.11 (d, $3J_{C-C-N-P/C-C-O-P} = 5.50$ Hz, OCH₂CH₂CH₂N), 26.78 (OCH₂C(CH₃)₃), 20.23 (2'CCH₃), 16.04 (CHCH₃).

HPLC (System 1) $t_R = 13.11$ min.

MS (EI+) m/z : 572.24 (MH⁺, 100%).

HRMS calculated for C₂₃H₃₇N₆O₇P₁: 572.2360, found 572.2352.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-(2*S*)-benzyl 2-(2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (7.4c).



Prepared according to the Standard Procedure 8, starting from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), (2*S*)-benzyl 2-(2-chloro-2-oxido-1,3,2-oxazaphosphinan-3-yl)propanoate (**7.2c**, 367 mg, 0.96 mmol), in 7 ml of dry pyridine. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product as white foam (**7.4c**, 34 mg, 6%).

³¹P NMR (202 MHz, MeOD) δ 9.15, 8.61.

¹H NMR (500 MHz, MeOD) δ 8.00 (s, 1H, H₈), 7.40-7.32 (m, 5H, OCH₂Ph), 6.00 (s, 1H, H_{1'}), 5.21, 5.16 (AB, $J_{AB}=12.5$ Hz, 2H, OCH₂Ph), 4.46-4.41 (m, 1H, CHCH₃), 4.38-4.29 (m, 5H, H_{3'}, H_{5'}, and OCH₂CH₂CH₂N), 4.23-4.22 (m, 1H, H_{4'}), 4.06 (s, 3H, 6OCH₃), 3.29-3.17 (m, 2H, OCH₂CH₂CH₂N), 2.10-2.03, 1.76-1.74 (m, 2H, OCH₂CH₂CH₂N), 1.42 (d, $J=7.5$ Hz, 3H, CHCH₃), 1.00 (s, 3H, 2'CCH₃).

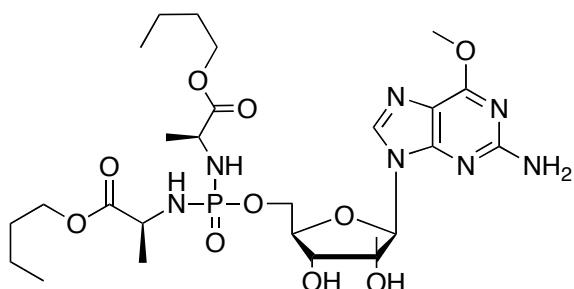
^{13}C NMR (126 MHz, MeOD) δ 173.46 (d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, C=O), 162.75 (C6), 161.98 (C2), 154.59 (C4), 138.91 (C8), 137.29 (ipso OCH_2Ph), 129.61, 129.50, 129.37 (OCH_2Ph), 115.52 (C5), 93.05 (C1'), 82.14 (d, $^3J_{\text{C-C-O-P}} = 7.6$ Hz, C4'), 80.05 (C2'), 74.42 (C3'), 71.02 (d, $^2J_{\text{C-O-P}} = 6.29$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$), 67.99 (OCH_2Ph), 66.35 (d, $^2J_{\text{C-N-P}} = 6.3$ Hz, C5'), 55.49 (d, $^2J_{\text{C-N-P}} = 3.77$ Hz, CHCH_3), 54.24 (6OCH_3), 44.15 ($\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$), 27.01 (d, $^3J_{\text{C-C-N-P}} = 6.2$ Hz, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{N}$), 20.25 ($2'\text{CCH}_3$), 16.00 (CHCH_3).

HPLC (System 1) $t_R = 13.09$ min.

MS (TOF ES+) m/z : 592.21 (MH^+ , 100%);

HRMS $\text{C}_{25}\text{H}_{33}\text{N}_6\text{O}_9\text{P}_1$ calculated: 592.2047 found: 592.2062

12.7 Experimental section – Chapter Eight.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(butoxy-L-alaninyl) phosphate (8.2b)

Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol),

Et₃N (0.11 ml, 0.80 mmol), L-alanine butyl ester hydrochloride salt (0.43 g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **8.2b** as a white foam (75 mg, 14%).

³¹P NMR (202 MHz, MeOD) δ 14.02.

¹H NMR (500 MHz, MeOD) δ 7.99 (s, 1H, H₈), 6.01 (s, 1H, H_{1'}), 4.40-4.37 (m, 2H, H_{5'}), 4.29 (d, J = 9.0 Hz, 1H, H_{3'}), 4.21- 4.19 (m, 1H, H_{4'}), 4.15- 4.01 (m, 7H, 6OCH₃ and 2x OCH₂CH₂CH₂CH₃), 3.94 (q, J =8.0 Hz, 2H, 2x CHCH₃), 1.63- 1.56 (m, 4 H, 2x OCH₂CH₂CH₂CH₃ ester), 1.41- 1.34 (m, 10H, 2x OCH₂CH₂CH₂CH₃ and 2x CHCH₃), 1.00 (s, 3H, 2'CCH₃), 0.93 (t, J = 7.5 Hz, 6H, 2x OCH₂CH₂CH₂CH₃).

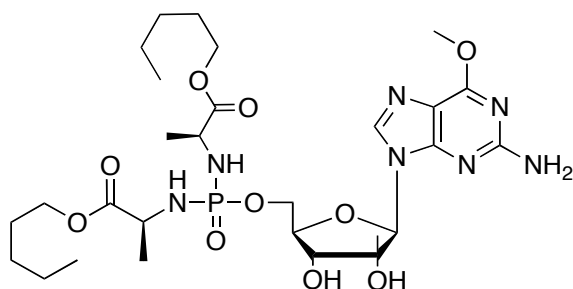
¹³C NMR (126 MHz, MeOD) δ 175.72, 175.65 (2d, ³J_{C-C-N-P}= 6.30 Hz, C=O), 162.74 (C6), 161.91 (C2), 154.60 (C4), 139.30 (C8), 115.55 (C5), 93.14 (C1'), 82.35 (d, ³J_{C-C-O-P}= 8.8 Hz, C4'), 80.06 (C2'), 74.81 (C3'), 66.22 (d, ²J_{C-O-P}= 5.0 Hz, C5'), 66.13 (OCH₂CH₂CH₂CH₃), 66.11 (OCH₂CH₂CH₂CH₃ ester), 54.26 (6OCH₃), 51.06 (d, ²J_{C-N-P}= 10.0 Hz, 2x CHCH₃), 31.75 (2x OCH₂CH₂CH₂CH₃ ester), 21.03, 20.86 (2d, ³J_{C-C-N-P}= 5.0 Hz, 2x CHCH₃), 20.33 (2'CCH₃), 20.13 (2x OCH₂CH₂CH₂CH₃), 14.07, 14.06 (2x OCH₂CH₂CH₂CH₃).

HPLC (System 1) t_R= 15.23 min.

MS (ES+) m/z: 668.28 (M+Na⁺, 100%).

HRMS C₂₆H₄₅N₇O₁₀P₁Na calculated: 646.2933 found: 646.2973.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(pentoxy-L-alaninyl) phosphate (8.2c).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-

alanine pentyl ester hydrochloride salt (0.79 g, 4.02 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **8.2c** as a white foam (260 mg, 40%).

³¹P NMR (202 MHz, MeOD) δ 14.05.

¹H NMR (500 MHz, MeOD) δ 8.00 (s, 1H, H₈), 6.03 (s, 1H, H_{1'}), 4.44-4.35 (m, 2H, H_{5'}), 4.29 (d, J = 8.5 Hz, 1H, H_{3'}), 4.22- 4.13 (m, 1H, H_{4'}), 4.13- 3.93 (m, 9H, 6OCH₃, 2x OCH₂CH₂CH₂CH₂CH₃ and 2x CHCH₃), 1.62-1.59 (m, 4H, 2x OCH₂CH₂CH₂CH₂CH₃), 1.38- 1.31 (m, 14H, 2x OCH₂CH₂CH₂CH₂CH₃, 2x OCH₂CH₂CH₂CH₂CH₃ and 2x CHCH₃), 1.00 (s, 3H, 2'CCH₃), 0.89 (t, J = 5.0 Hz, 6H, 2x OCH₂CH₂CH₂CH₂CH₃).

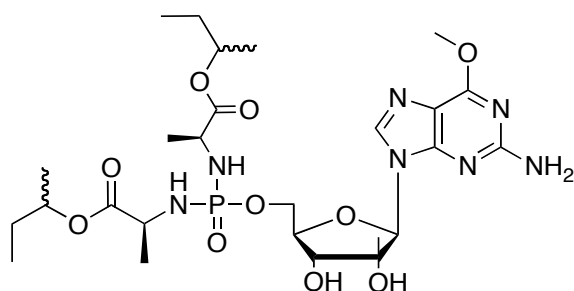
¹³C NMR (126 MHz, MeOD) δ 175.72, 175.67 (2d, ³ $J_{C-C-N-P}$ = 6.30 Hz, C=O), 162.75 (C6), 161.92 (C2), 154.61 (C4), 139.26 (C8), 115.56 (C5), 93.07 (C1'), 82.34 (d, ³ $J_{C-C-O-P}$ = 7.5 Hz, C4'), 80.11 (C2'), 74.81 (C3'), 66.43 (d, ² J_{C-O-P} = 2.52 Hz, C5'), 66.25, 66.22 (2x OCH₂CH₂CH₂CH₂CH₃ ester), 54.34 (6OCH₃), 51.16 (d, ² J_{C-N-P} = 11.3 Hz, CHCH₃), 51.07 (d, ² J_{C-N-P} = 10.0 Hz, CHCH₃), 29.39 (2x OCH₂CH₂CH₂CH₂CH₃ ester), 29.16 (2x OCH₂CH₂CH₂CH₂CH₃ ester), 23.38 (2x OCH₂CH₂CH₂CH₂CH₃ ester), 21.12, 21.01 (2d, ³ $J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.43 (2'CCH₃), 14.07, 14.42 (2x OCH₂CH₂CH₂CH₂CH₃).

HPLC t_R = 18.68 min.

MS (ES+) m/z : 674.32 (M+H⁺, 100%).

HRMS C₂₈H₄₈N₇O₁₀P₁ Calculated: 674.3279, found: 674.3246.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(*R,S*-2-butoxy-L-alaninyl) phosphate (8.2d).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-

alanine *R,S*-2-butyl ester hydrochloride salt (0.73 g, 4.02 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **8.2d** as a white foam (90 mg, 17%).

³¹P NMR (202 MHz, MeOD) 14.05.

¹H NMR (500 MHz, MeOD) 7.99 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.80- 4.78 (m, 2H, 2x OCH(CH₃)CH₂CH₃), 4.41- 4.39 (m, 2H, H_{5'}), 4.30 (d, *J* = 8.5 Hz, 1H, H_{3'}), 4.21- 4.20 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.92-3.90 (m, 2H, 2x CHCH₃), 1.63- 1.51 (m, 4H, 2x OCH(CH₃)CH₂CH₃), 1.37- 1.33 (m, 6H, 2x CHCH₃), 1.24- 1.17 (m, 6H, 2x OCH(CH₃)CH₂CH₃), 1.00 (s, 3H, 2'CCH₃), 0.89 (t, *J* = 6.0 Hz, 2x OCH(CH₃)CH₂CH₃).

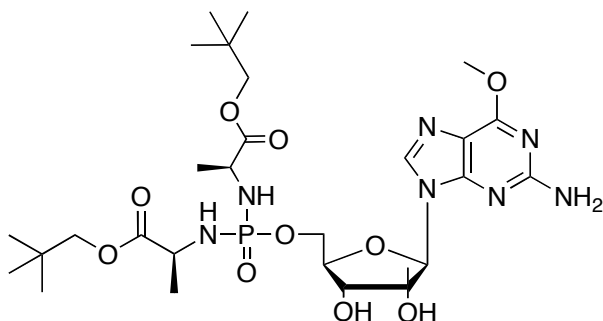
¹³C-NMR (126 MHz, MeOD) 175.30, 175.16 (2d, ³*J*_{C-C-N-P} = 3.8 Hz, C=O), 162.74 (C6), 161.89 (C2), 154.58 (C4), 139.37 (C8), 115.58 (C5), 93.19 (C1'), 82.38, 82.36 (d, ³*J*_{C-C-O-P} = 7.6 Hz, C4'), 80.06 (C2'), 74.91 (C3'), 74.59, 74.55 (OCH(CH₃)CH₂CH₃), 66.40 (d, ²*J*_{C-O-P} = 3.8 Hz, C5'), 54.27 (6OCH₃), 51.19 (d, ²*J*_{C-N-P} = 7.6 Hz, CHCH₃), 51.14 (d, ²*J*_{C-N-P} = 8.8 Hz, CHCH₃), 29.78, 29.74 (OCH(CH₃)CH₂CH₃), 21.17 (d, ³*J*_{C-C-N-P} = 6.2 Hz, CHCH₃), 21.04 (d, ³*J*_{C-C-N-P} = 2.50 Hz, CHCH₃), 21.01 (CHCH₃), 20.91 (d, ³*J*_{C-C-N-P} = 5.25 Hz, CHCH₃), 20.89 (2'CCH₃), 19.76, 19.61 (OCH(CH₃)CH₂CH₃), 10.05 (OCH(CH₃)CH₂CH₃).

HPLC *t*_R = 15.12 min.

MS (ES+) *m/z*: 668.28 (M+Na⁺, 100%).

HRMS C₂₆H₄₄N₇O₁₀P₁ calculated: 646.2966 found: 646.2961.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (8.2e).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 1.00g, 3.18 mmol), POCl₃ (0.28 ml, 3.18 mmol), Et₃N (0.43 ml, 3.18 mmol), L-alanine 2,2-dimethylpropyl ester

tosylate salt (**3.2g**, 5.30 g, 16.05 mmol), Et₃N (2.68 ml, 32.10 mmol) in 40 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **8.2e** as a white foam (640 mg, 30%).

³¹P NMR (202 MHz, MeOD) δ 14.01.

¹H NMR (500 MHz, MeOD) δ 7.98 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.41-4.37 (m, 2H, H_{5'}), 4.28 (d, 1H, *J*=9.0 Hz, H_{3'}), 4.22-4.16 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 4.05–3.93 (m, 2H, 2x CHCH₃), 3.86, 3.84, 3.82, 3.79 (2AB, *J*_{AB}= 10.0 Hz, 2H, 2x OCH₂C(CH₃)₃), 1.40, 1.36 (2d, 6H, *J*=7.5 Hz, 2x CHCH₃), 0.99 (s, 3H, 2CCH₃), 0.95, 0.94 (2s, 18H, 2x OCH₂C(CH₃)₃).

¹³C-NMR (126 MHz, MeOD) 175.30 (2d, ³*J*_{C-C-N-P}= 3.8 Hz, C=O), 162.74 (C6), 161.90 (C2), 154.57 (C4), 139.37 (C8), 115.60 (C5), 93.20 (C1'), 82.37 (d, ³*J*_{C-C-O-P}= 7.6 Hz, C4'), 80.06 (C2'), 75.72, 75.64 (2x OCH₂C(CH₃)₃), 74.89 (C3'), 74.59, 74.55 (OCH(CH₃)CH₂CH₃), 66.38 (d, ²*J*_{C-O-P}= 3.8 Hz, C5'), 54.30 (6OCH₃), 51.20, 51.17 (2d, ²*J*_{C-N-P}= 7.6 Hz, CHCH₃), 32.14, 32.09 (2x OCH₂C(CH₃)₃), 27.08, 26.98 (2x OCH₂C(CH₃)₃), 21.15 (d, ³*J*_{C-C-N-P}= 6.0 Hz, CHCH₃), 21.05 (d, ³*J*_{C-C-N-P}= 5.3 Hz, CHCH₃), 20.90 (2'CCH₃).

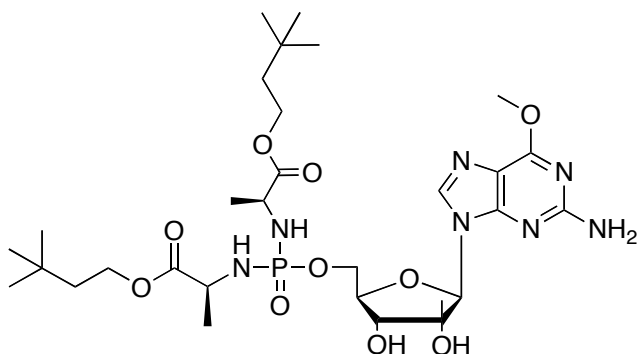
HPLC (System 1) *t*_R= 16.95 min.

HPLC (System 2) *t*_R= 27.95 min.

MS (ES+) *m/z*: 673.32 (M+H⁺, 100%).

HRMS C₂₆H₄₉N₇O₁₀P₁ calculated: 673.3271 found: 673.3215.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(3,3-dimethylbutoxy-L-alaninyl) phosphate (8.2f).



Prepared according to the standard procedure 13a from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-alanine 3,3-dimethylbutyl ester

tosylate salt (**3.2i**, 0.83 g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. Crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5% gradient), to give a pure product **8.2f** as a white foam (52 mg, 9%).

³¹P NMR (202 MHz, MeOD) δ 14.03.

¹H NMR (500 MHz, MeOD) δ 7.99 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.42-4.36 (m, 2H, H_{5'}), 4.28 (d, J = 9.0 Hz, 1H, H_{3'}), 4.23- 4.09 (m, 5H, H_{4'} and 2x OCH₂CH₂C(CH₃)₃), 4.07 (s, 3H, 6OCH₃), 3.92 (q, J = 7.0 Hz, 2H, 2x CHCH₃), 1.60 (t, J = 7.0 Hz, 4H, 2x OCH₂CH₂C(CH₃)₃), 1.37 (d, J = 7.0 Hz, 6H, 2x CHCH₃), 0.99 (s, 3H, 2'CCH₃), 0.97 (s, 9H, OCH₂CH₂C(CH₃)₃), 0.94 (s, 9H, OCH₂CH₂C(CH₃)₃).

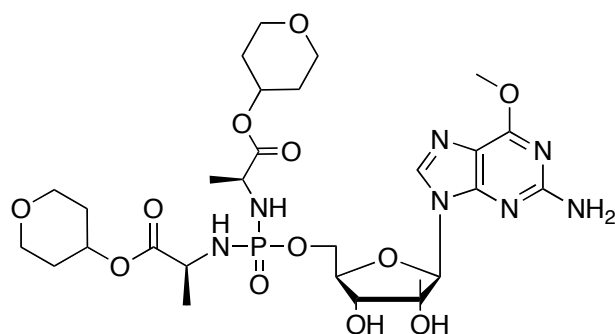
¹³C NMR (126 MHz, MeOD) δ 175.67, 175.59 (2d, ³ $J_{C-C-N-P}$ = 6.3 Hz, C=O), 162.74 (C6), 161.91 (C2), 154.61 (C4), 139.24 (C8), 115.58 (C5), 93.13 (C1'), 82.35 (d, ³ $J_{C-C-O-P}$ = 8.8 Hz, C4'), 80.06 (C2'), 74.75 (C3'), 66.22 (d, ² J_{C-O-P} = 5.0 Hz, C5'), 66.96 (OCH₂CH₂C(CH₃)₃), 66.94 (OCH₂CH₂C(CH₃)₃), 54.24 (6OCH₃), 51.07 (d, ² J_{C-N-P} = 10.0 Hz, 2x CHCH₃), 42.85 (OCH₂CH₂C(CH₃)₃), 42.83 (OCH₂CH₂C(CH₃)₃), 30.59 (OCH₂CH₂C(CH₃)₃), 30.55 (OCH₂CH₂C(CH₃)₃), 30.03 (2x OCH₂CH₂C(CH₃)₃), 20.96 (d, ³ $J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.79 (d, ³ $J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.34 (2'CCH₃).

HPLC (System 1) t_R = 20.12 min.

MS (ES⁺) m/z : 724.35 (M+Na⁺, 100%).

HRMS C₃₀H₅₃N₇O₁₀P₁ calculated: 702.3592 found: 702.3621.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(3,3-dimethylbutoxy-L-alaninyl) phosphate (8.2h).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80

mmol), L-alanine tetrahydropyranyl ester tosylate salt (0.83g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), to give a pure product **8.2h** as a white foam (75 mg, 14%).

³¹P NMR (202 MHz, MeOD) δ 13.94.

¹H NMR (500 MHz, MeOD) δ 7.99 (s, 1H, H₈), 5.99 (s, 1H, H_{1'}), 4.94- 4.83 (m, 2H, 2x OCH ester), 4.39-4.38 (m, 2H, H_{5'}), 4.28 (d, J = 9.0 Hz, 1H, H_{3'}), 4.21- 4.18 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.99- 3.83 (m, 6H, 2x CHCH₃ and 4x OCH_{2a} ester), 3.56- 3.45 (m, 4H, 4x OCH_{2b} ester), 1.93- 1.83 (m, 4 H, 4x OCH_{2c} ester), 1.69- 1.59 (m, 4 H, 4x OCH_{2d} ester), 1.38 (d, J = 7.0 Hz, 3H, CHCH₃), 1.36 (d, J = 7.0 Hz, 3H, CHCH₃), 0.99 (s, 3H, 2'CCH₃).

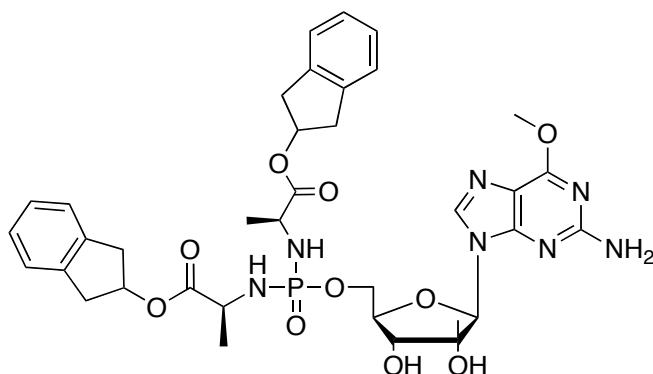
¹³C NMR (126 MHz, MeOD) δ 175.00, 174.92 (2d, ³ $J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.75 (C6), 161.94 (C2), 154.63 (C4), 139.36 (C8), 115.54 (C5), 93.14 (C1'), 82.41 (d, ³ $J_{C-C-O-P}$ = 8.8 Hz, C4'), 80.03 (C2'), 74.89 (C3'), 71.27 (OCH ester), 71.23 (OCH ester), 66.47 (d, ² J_{C-O-P} = 5.0 Hz, C5'), 66.18 (4x OCH₂ ester), 54.29 (6OCH₃), 51.15 (d, ² J_{C-N-P} = 6.3 Hz, 2x CHCH₃), 32.64 (4x CH₂ ester), 20.98 (d, ³ $J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.79 (d, ³ $J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.31 (2'CCH₃).

HPLC (System 1) t_R = 9.57 min.

MS (ES+) m/z : 724.27 (M+Na⁺, 100%).

HRMS C₂₈H₄₅N₇O₁₂P₁ calculated: 702.2864 found: 702.2848.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(indanyl-L-alaninyl) phosphate (8.2j).



Prepared according to the standard procedure 13a, from:

2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-alanine 2-indanyl

ester tosylate salt (**3.2k**, 0.91 g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2j** was obtained as a white foam (52 mg, 9%).

³¹P NMR (202 MHz, MeOD) δ 13.94.

¹H NMR (500 MHz, MeOD) δ 7.95 (s, 1H, H₈), 7.19-7.10 (m, 8H, 8 x CH-Ar), 5.99 (s, 1H, H_{1'}), 5.49- 5.46 (m, 1H, OCH ester), 5.42- 5.40 (m, 1H, OCH ester), 4.34-4.25 (m, 3H, H_{5'} and H_{3'}), 4.16- 4.14 (m, 1H, H_{4'}), 4.03 (s, 3H, 6OCH₃), 3.83 (q, J =7.0 Hz, 2H, 2x CHCH₃), 3.28-3.16 (m, 4H, 2x CH₂ ester), 3.01- 2.90 (m, 4H, 2x CH₂ ester), 1.25 (d, J = 7.0 Hz, 3H, CHCH₃), 1.24 (d, J = 7.0 Hz, 3H, CHCH₃), 0.98 (s, 3H, 2'CCH₃).

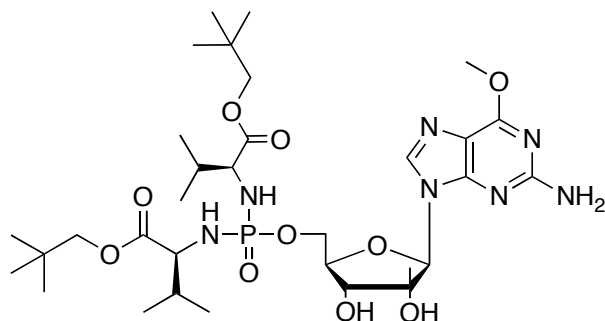
¹³C NMR (126 MHz, MeOD) δ 175.53, 175.48 (2d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.73 (C6), 161.89 (C2), 154.57 (C4), 141.59, 141.51, 141.47 (C-Ar), 139.30 (C8), 127.84, 125.59 (CH-Ar), 115.58 (C5), 93.13 (C1'), 82.35 (d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 80.04 (C2'), 77.70 (OCH ester), 77.67 (OCH ester), 74.81 (C3'), 66.28 (d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 54.29 (6OCH₃), 51.11 (d, $^2J_{C-N-P}$ = 10.0 Hz, 2x CHCH₃), 40.42, 40.36 (CH₂ ester), 20.76 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.62 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.38 (2'CCH₃).

HPLC (System 1) t_R = 17.88 min.

MS (ES+) m/z : 788.28 (M+Na⁺, 100%).

HRMS C₃₆H₄₅N₇O₁₀Na₁P₁ calculated: 788.2785 found: 788.2771.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropyl-L-valinyl) phosphate (8.2k).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), L-valine 2,2-dimethylpropyl ester

tosylate salt (1.98 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2k** was obtained as a white foam (42 mg, 6%).

³¹P NMR (202 MHz, MeOD) δ 15.16.

¹H NMR (500 MHz, MeOD) δ 7.99 (s, 1H, H₈), 5.99 (s, 1H, H_{1'}), 4.41-4.35 (m, 2H, H_{5'}), 4.33 (d, $J = 9.0$ Hz, 1H, H_{3'}), 4.20- 4.17 (m, 1H, H_{4'}), 4.09 (s, 3H, 6OCH₃), 3.87-3.68 (m, 6H, 2x CHCH(CH₃)₂ and 2x OCH₂C(CH₃)₃), 2.11-1.98 (m, 2H, 2x CHCH(CH₃)₂), 0.99, 0.98 (2s, 3H, 2'CCH₃), 0.95, 0.94 (2s, 18H, 2x OCH₂C(CH₃)₃), 0.89- 0.83 (m, 12H, 2x CHCH(CH₃)₂).

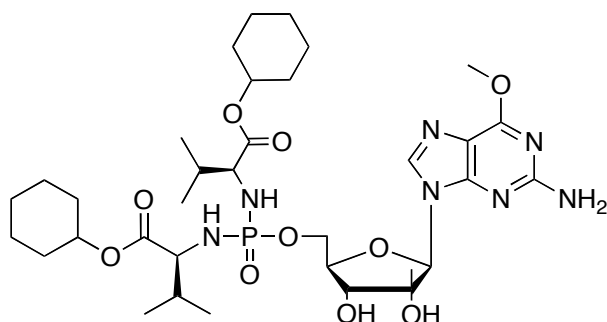
¹³C NMR (126 MHz, MeOD) δ 174.87, 174.80 (2d, $^3J_{C-C-N-P} = 2.5$ Hz, 2x C=O), 162.77 (C6), 161.92 (C2), 154.54 (C4), 139.49 (C8), 115.68 (C5), 93.35 (C1'), 82.55 (d, $^3J_{C-C-O-P} = 8.8$ Hz, C4'), 79.92 (C2'), 75.65 (OCH₂C(CH₃)₃), 75.58 (OCH₂C(CH₃)₃), 75.03 (C3'), 67.11 (d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 61.09 (CHCH(CH₃)₂), 61.03 (CHCH(CH₃)₂), 54.24 (6OCH₃), 33.23 (d, $^3J_{C-C-N-P} = 6.3$ Hz, 2x CHCH(CH₃)₂), 32.14 (2x OCH₂C(CH₃)₃), 32.08 (2x OCH₂C(CH₃)₃), 26.89 (2x OCH₂C(CH₃)₃), 20.32 (2'CCH₃), 18.09 (CHCH(CH₃)₂), 18.07 (CHCH(CH₃)₂).

HPLC(System 1) t_R = 22.15 min.

MS (ES+) m/z: 752.37 (M+Na⁺, 100%).

HRMS C₃₂H₅₆N₇O₁₀Na₁P₁ calculated: 752.3724 found: 752.3736.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(cyclohexoxy-L-valinyl) phosphate (8.21).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-valine cyclohexyl ester

hydrochloride salt (0.57 g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.21** was obtained as a white foam (32 mg, 5%).

³¹P NMR (202 MHz, MeOD) δ 15.28.

¹H NMR (500 MHz, MeOD) δ 7.96 (s, 1H, H₈), 5.98 (s, 1H, H_{1'}), 4.81-4.76 (m, 2H, 2x OCH ester), 4.41-4.35 (m, 2H, H_{5'}), 4.33 (d, J = 9.0 Hz, 1H, H_{3'}), 4.20- 4.17 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.66-3.63 (m, 2H, 2x CHCH(CH₃)₂), 2.07-1.98 (m, 2H, 2x CHCH(CH₃)₂), 1.85- 1.80 (m, 4H, 2x CH₂ ester), 1.75- 1.71 (m, 4H, 2x CH₂ ester), 1.55- 1.29 (m, 12H, 6x CH₂ ester), 1.00 (s, 3H, 2'CCH₃), 0.95 (d, J = 6.5 Hz, 3H, CHCH(CH₃)₂), 0.90 (d, J = 6.5 Hz, 3H, CHCH(CH₃)₂), 0.89 (d, J = 6.5 Hz, 3H, CHCH(CH₃)₂), 0.82 (d, J = 6.5 Hz, 3H, CHCH(CH₃)₂).

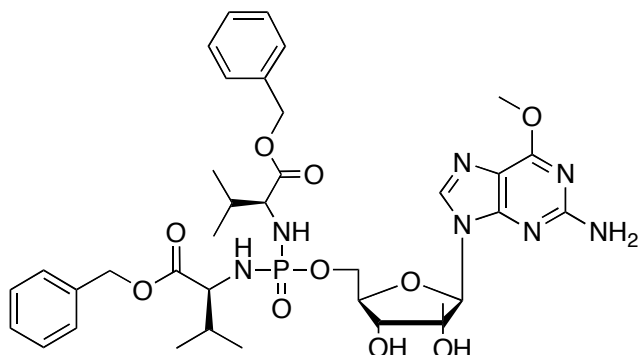
¹³C NMR (126 MHz, MeOD-*d*4) δ 174.12 (d, ³ $J_{C-C-N-P}$ = 5.0 Hz, C=O), 162.77 (C6), 161.94 (C2), 154.56 (C4), 139.47 (C8), 115.66 (C5), 93.34 (C1'), 82.55 (d, ³ $J_{C-C-O-P}$ = 6.3 Hz, C4'), 79.91 (C2'), 75.02 (C3'), 74.86 (OCH ester), 74.84 (OCH ester), 67.06 (d, ² J_{C-O-P} = 5.0 Hz, C5'), 61.04 (d, ² J_{C-N-P} = 4.5 Hz, 2x CHCH(CH₃)₂), 54.22 (6OCH₃), 33.21 (2x CHCH(CH₃)₂), 32.60 (CH₂ ester), 26.43 (CH₂ ester), 26.40 (CH₂ ester), 24.68 (CH₂ ester), 24.64 (CH₂ ester), 20.27 (2'CCH₃), 19.48 (CHCH(CH₃)₂), 19.37 (CHCH(CH₃)₂), 18.08 (CHCH(CH₃)₂), 18.02 (CHCH(CH₃)₂).

HPLC (System 1) t_R = 22.99 min.

MS (ES+) m/z : 776.40 (M+Na⁺, 100%).

HRMS C₃₈H₆₀N₇O₁₀P₁ calculated: 754.3905 found: 754.3906.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(benzyloxy-L-valinyl) phosphate (8.2m).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (250 mg, 0.80 mmol), POCl₃ (0.07 ml, 0.80 mmol), Et₃N (0.11 ml, 0.80 mmol), L-valine benzyl ester tosylate salt

(0.91 g, 2.41 mmol), Et₃N (0.67 ml, 4.82 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2m** was obtained as a white foam (64 mg, 10%).

³¹P NMR (202 MHz, MeOD) δ 15.11.

¹H NMR (500 MHz, MeOD) δ 7.94 (s, 1H, H₈), 7.34-7.25 (m, 10H, 2x OCH₂Ph), 5.98 (s, 1H, H_{1'}), 5.19- 5.06 (m, 4H, 2x OCH₂Ph), 4.40-4.34 (m, 2H, H_{5'} and H_{3'}), 4.20- 4.18 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.72-3.68 (m, 2H, 2x CHCH(CH₃)₂), 2.02-1.94 (m, 2H, 2x CHCH(CH₃)₂), 0.99 (s, 3H, 2'CCH₃), 0.87 (d, *J*= 7.0 Hz, 3H, CHCH(CH₃)₂), 0.84 (d, *J*= 7.0 Hz, 3H, CHCH(CH₃)₂), 0.79(d, *J*= 7.0 Hz, 3H, CHCH(CH₃)₂), 0.77 (d, *J*= 7.0 Hz, 3H, CHCH(CH₃)₂).

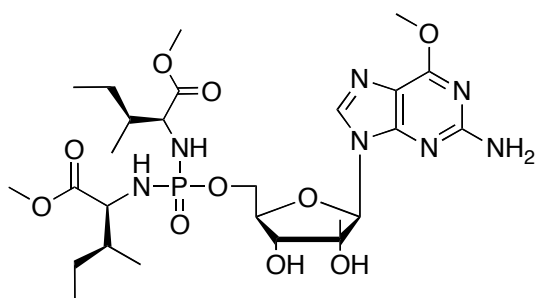
¹³C NMR (126 MHz, MeOD) δ 174.53 (C=O), 162.75 (C6), 161.90 (C2), 154.54 (C4), 139.46 (C8), 137.21 (ipso OCH₂Ph), 137.14 (ipso OCH₂Ph), 129.61, 129.59, 129.58, 129.55, 129.41, 129.36 (2x OCH₂Ph), 115.68 (C5), 93.29 (C1'), 82.49 (d, ³*J*_{C-O-P} = 8.8 Hz, C4'), 79.94 (C2'), 74.97 (C3'), 67.93 (2x CH₂Ph), 66.89 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 61.09 (d, ²*J*_{C-N-P} = 4.5 Hz, 2x CHCH(CH₃)₂), 54.27 (6OCH₃), 33.20 (d, ³*J*_{C-C-N-P} = 6.3 Hz, 2x CHCH(CH₃)₂), 20.37 (2'CCH₃), 19.50 (CHCH(CH₃)₂), 19.42 (CHCH(CH₃)₂), 18.17 (CHCH(CH₃)₂), 18.12 (CHCH(CH₃)₂).

HPLC(System 1) *t*_R= 19.63 min.

MS (ES+) *m/z*: 770.33 (M+H⁺, 100%).

HRMS C₃₆H₄₉N₇O₁₀P₁ calculated: 770.3279 found: 770.3311.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(methoxy-L-isoleucinyl) phosphate (8.2n).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), L-leucine methyl ester hydrochloride salt

(1.15 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2n** was obtained as a white foam (135 mg, 22%).

³¹P NMR (202 MHz, MeOD) δ 14.88.

¹H NMR (500 MHz, MeOD) δ 8.01 (s, 1H, H₈), 5.99 (s, 1H, H_{1'}), 4.47-4.38 (m, 2H, H_{5'}), 4.34 (d, J = 9.0 Hz, 1H, H_{3'}), 4.20- 4.18 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.87-3.83 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 3.72, 3.70 (2s, 6H, OCH₃ ester), 1.74-1.69 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 1.44-1.36 and (m, 2H, CHCH(CH₃)CH₂CH₃), 1.09-1.03 (m, 2H, CHCH(CH₃)CH₂CH₃), 1.00 (s, 3H, 2'CCH₃), 0.91 (d, J = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.89 (d, J = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.84 (t, J = 7.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.82 (t, J = 7.5 Hz, 3H, CHCH(CH₃)CH₂CH₃).

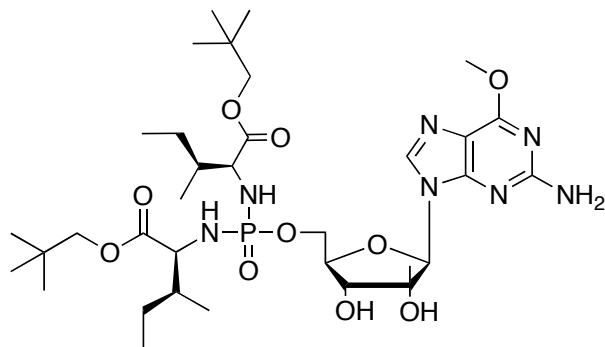
¹³C NMR (126 MHz, MeOD) δ 175.17 (d, $^3J_{C-C-N-P}$ = 3.8 Hz, 2x C=O), 162.74 (C6), 161.91 (C2), 154.46 (C4), 139.40 (C8), 115.44 (C5), 93.27 (C1'), 82.51 (d, $^3J_{C-C-O-P}$ = 7.5 Hz, C4'), 79.97 (C2'), 74.87 (C3'), 66.72 (d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 60.02 (d, $^2J_{C-N-P}$ = 4.5 Hz, CHCH(CH₃)CH₂CH₃), 60.00 (d, $^2J_{C-N-P}$ = 4.5 Hz, CHCH(CH₃)CH₂CH₃), 54.37 (6OCH₃), 52.54, 52.51 (2x OCH₃ ester), 33.21 (d, $^3J_{C-C-N-P}$ = 3.8 Hz CHCH(CH₃)CH₂CH₃), 33.19 (d, $^3J_{C-C-N-P}$ = 7.5 Hz CHCH(CH₃)CH₂CH₃), 26.10 (CHCH(CH₃)CH₂CH₃), 26.08 (CHCH(CH₃)CH₂CH₃), 20.41 (2'CCH₃), 15.91 (CHCH(CH₃)CH₂CH₃), 15.83 (CHCH(CH₃)CH₂CH₃), 11.93 (CHCH(CH₃)CH₂CH₃).

HPLC (System 1) t_R = 10.87 min.

MS (ES⁺) *m/z*: 668.28 (M+Na⁺, 100%).

HRMS C₂₆H₄₄N₇O₁₀Na₁P₁ calculated: 668.2785 found: 668.2759.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropoxy-L-isoleuciny) phosphate (8.2o).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol),

L-leucine 2,2-dimethylpropyl ester tosylate salt (**3.2p**, 1.80 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2o** was obtained as a white foam (210 mg, 29%).

³¹P NMR (202 MHz, MeOD) δ 14.93.

¹H NMR (500 MHz, MeOD-*d*₄) δ 7.96 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.41-4.40 (m, 2H, H_{5'}), 4.34 (d, *J* = 8.5 Hz, 1H, H_{3'}), 4.21- 4.20 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.84-3.72 (m, 6H, 2x OCH₂C(CH₃)₃ and 2x CHCH(CH₃)CH₂CH₃), 1.81-1.78 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 1.46-1.41, 1.39-1.33 (2m, 2H, CHCH(CH₃)CH₂CH₃), 1.16-1.11, 1.07-1.04 (2m, 2H, CHCH(CH₃)CH₂CH₃), 1.00 (s, 3H, 2'CCH₃), 0.95, 0.94 (2s, 2x OCH₂C(CH₃)₃), 0.91 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.88 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.83 (t, *J* = 7.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.81 (t, *J* = 7.5 Hz, 3H, CHCH(CH₃)CH₂CH₃).

¹³C NMR (126 MHz, MeOD-*d*₄) δ 174.79, 174.72 (2d, ³*J*_{C-C-N-P} = 3.8 Hz, 2x C=O), 162.79 (C6), 161.90 (C2), 154.58 (C4), 139.46 (C8), 115.70 (C5), 93.28 (C1'), 82.56 (d, ³*J*_{C-C-O-P} = 6.3 Hz, C4'), 79.97 (C2'), 75.72, 75.64 (2x OCH₂C(CH₃)₃), 74.98 (C3'), 67.00 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 60.17 (CHCH(CH₃)CH₂CH₃), 60.07 (CHCH(CH₃)CH₂CH₃), 54.32 (6OCH₃), 40.30 (CHCH(CH₃)CH₂CH₃), 40.25

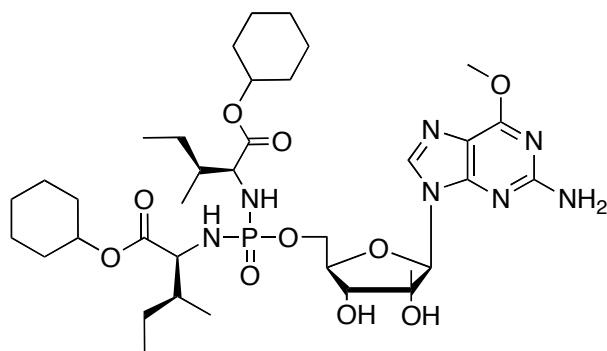
(CHCH(CH₃)CH₂CH₃), 32.14 (OCH₂C(CH₃)₃), 32.09 (OCH₂C(CH₃)₃), 27.08 (OCH₂C(CH₃)₃), 26.98 (OCH₂C(CH₃)₃), 26.04 (2x CHCH(CH₃)CH₂CH₃), 20.43 (2'CCH₃), 16.14 (CHCH(CH₃)CH₂CH₃), 16.03 (CHCH(CH₃)CH₂CH₃), 12.13 (CHCH(CH₃)CH₂CH₃), 12.09 (CHCH(CH₃)CH₂CH₃).

HPLC (System 1) *t_R* = 24.55 min.

MS (ES+) *m/z*: 780.40 (M+Na⁺, 100%).

HRMS C₃₄H₆₁N₇O₁₀P₁ calculated: 758.4218 found: 758.4196.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-bis(cyclohexoxy-L-isoleuciny) phosphate (8.2p).



Prepared according to the standard procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), L-leucine cyclohexyl ester tosylate

salt (**3.2o**, 1.86 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2p** was obtained as a white foam (140 mg, 19%).

³¹P NMR (202 MHz, MeOD) δ 15.04.

¹H NMR (500 MHz, MeOD) δ 7.97 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.80-4.74 (m, 2H, 2x OCH ester), 4.43-4.39 (m, 2H, H_{5'}), 4.33 (d, *J* = 9.0 Hz, 1H, H_{3'}), 4.22- 4.19 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.74-3.73 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 1.82- 1.80 (m, 4H, 2x CH₂ ester), 1.75- 1.70 (m, 6H, 2x CH₂ ester and 2x CHCH(CH₃)CH₂CH₃), 1.55- 1.29 (m, 14H, 6x CH₂ ester and CHCH(CH₃)CH₂CH₃), 1.15-1.02 (m, 2H, CHCH(CH₃)CH₂CH₃), 1.00 (s, 3H, 2'CCH₃), 0.91 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.88 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.85 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃), 0.83 (d, *J* = 6.5 Hz, 3H, CHCH(CH₃)CH₂CH₃).

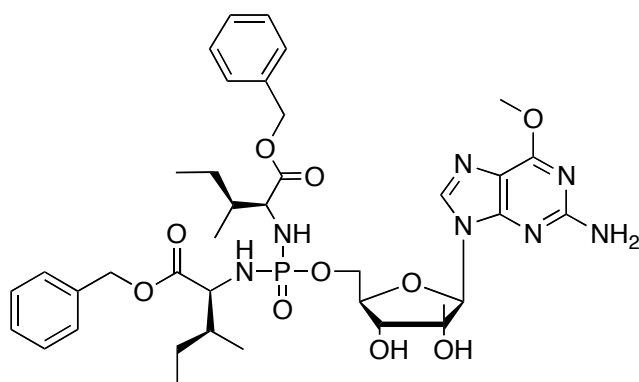
^{13}C NMR (126 MHz, MeOD) δ 174.05 (d, $^3J_{\text{C-C-N-P}} = 3.8$ Hz, C=O), 174.03 (d, $^3J_{\text{C-C-N-P}} = 3.8$ Hz, C=O), 162.79 (C6), 161.91 (C2), 154.59 (C4), 139.44 (C8), 115.69 (C5), 93.26 (C1'), 82.54 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 79.97 (C2'), 74.98 (C3'), 74.82 (2x OCH ester), 66.11 (d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 60.02 (2x CHCH(CH₃)CH₂CH₃), 54.33 (6OCH₃), 40.30 (4x CH₂ ester), 32.64 (d, $^3J_{\text{C-C-N-P}} = 3.8$ Hz, CHCH(CH₃)CH₂CH₃), 32.60 (d, $^3J_{\text{C-C-N-P}} = 7.5$ Hz, CHCH(CH₃)CH₂CH₃), 26.47 (CH₂ ester), 26.44 (CH₂ ester), 26.09 (2x CHCH(CH₃)CH₂CH₃), 24.69 (CH₂ ester), 24.67 (CH₂ ester), 20.42 (2'CCH₃), 16.01 (CHCH(CH₃)CH₂CH₃), 15.91 (CHCH(CH₃)CH₂CH₃), 12.14 (CHCH(CH₃)CH₂CH₃), 12.08 (CHCH(CH₃)CH₂CH₃).

HPLC (System 1) $t_{\text{R}} = 25.45$ min.

MS (ES⁺) m/z : 804.41 (M+Na⁺, 100%);

HRMS C₃₆H₆₁N₇O₁₀P₁ calculated: 782.4218 found: 782.4234

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(benzyloxy-L-isoleuciny) phosphate (8.2q).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), L-leucine benzyl

ester tosylate salt (1.89 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2q** was obtained as a white foam (200 mg, 16%).

^{31}P NMR (202 MHz, MeOD) δ 14.93.

^1H NMR (500 MHz, MeOD) δ 7.92 (s, 1H, H₈), 7.42-7.28 (m, 10H, 2x OCH₂Ph), 5.99 (s, 1H, H_{1'}), 5.21-5.18 (m, 4H, 2x OCH₂Ph), 4.43-4.40 (m, 2H, H_{5'}), 4.37 (d, $J = 8.5$ Hz, 1H, H_{3'}), 4.19-4.15 (m, 1H, H_{4'}), 4.03 (s, 3H, 6OCH₃), 3.74-3.73 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 1.75-1.73 (m, 2H, 2x CHCH(CH₃)CH₂CH₃), 1.37-1.29 (m,

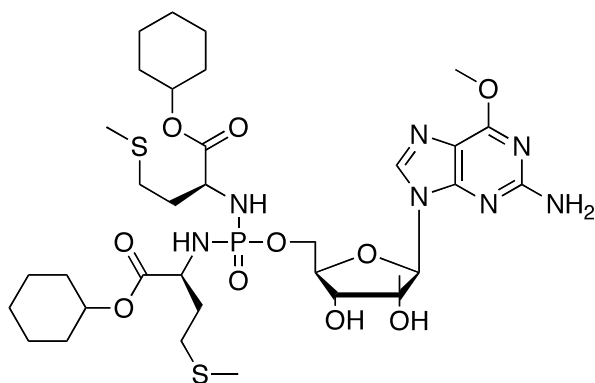
2H, CHCH(CH₃)CH₂CH₃), 1.15-1.02 (m, 2H, CHCH(CH₃)CH₂CH₃), 1.00 (s, 3H, 2'CCH₃), 0.83-0.69 (m, 12H, 2x CHCH(CH₃)CH₂CH₃ and 2x CHCH(CH₃)CH₂CH₃).
¹³C NMR (126 MHz, MeOD) δ 174.47 (2x C=O), 162.77 (C6), 161.90 (C2), 154.58 (C4), 139.42 (C8), 137.19 (ipso OCH₂Ph), 137.12 (ipso OCH₂Ph), 129.93, 129.59, 129.42, 129.39 (2x OCH₂Ph), 115.68 (C5), 93.22 (C1'), 82.49 (d, ³J_{C-C-O-P} = 7.5 Hz, C4'), 79.95 (C2'), 74.91 (C3'), 67.91 (OCH₂Ph), 67.75 (OCH₂Ph), 66.77 (d, ²J_{C-O-P} = 5.0 Hz, C5'), 60.19 (CHCH(CH₃)CH₂CH₃), 60.10 (CHCH(CH₃)CH₂CH₃), 54.31 (6OCH₃), 40.16 (d, ³J_{C-C-N-P} = 3.8 Hz, CHCH(CH₃)CH₂CH₃), 40.11 (d, ³J_{C-C-N-P} = 3.8 Hz, CHCH(CH₃)CH₂CH₃), 25.96 (CHCH(CH₃)CH₂CH₃), 25.92 (CHCH(CH₃)CH₂CH₃), 20.42 (2'CCH₃), 15.87(CHCH(CH₃)CH₂CH₃), 15.74 (CHCH(CH₃)CH₂CH₃), 11.95 (CHCH(CH₃)CH₂CH₃), 11.92 (CHCH(CH₃)CH₂CH₃).

HPLC (System 1) t_R = 20.88 min.

MS (ES+) m/z: 820.34 (M+Na⁺, 100%).

HRMS C₃₈H₅₂N₇O₁₀Na₁P₁ calculated: 820.3411 found: 820.3378.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-bis(cyclohexoxy-*L*-methioninyl) phosphate (8.2s).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), *L*-methionine cyclohexyl ester tosylate

salt (**3.21**, 1.94 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2s** was obtained as a white foam (160 mg, 20%).

³¹P NMR (202 MHz, MeOD) δ 14.26.

¹H NMR (500 MHz, MeOD) δ 8.07 (s, 1H, H₈), 6.00 (s, 1H, H_{1'}), 4.79-4.74 (m, 1H, OCH ester), 4.73-4.70 (m, 1H, OCH ester), 4.45-4.36 (m, 2H, H_{5'}), 4.30 (d, 1H, *J*=

8.5 Hz, H_{3'}), 4.23-4.20 (m, 1H, H_{4'}), 4.08 (s, 3H, 6OCH₃), 4.02-3.97 (m, 2H, 2x CHCH₂CH₂SCH₃), 2.61-2.48 (m, 4H, 2x CHCH₂CH₂SCH₃), 2.07, 2.05 (2s, 6H, 2x CHCH₂CH₂SCH₃), 2.02-1.87 (m, 4H, 2x CHCH₂CH₂SCH₃), 1.86- 1.79 (m, 4H, 2x CH₂ ester), 1.73- 1.71 (m, 4H, 2x CH₂ ester), 1.47- 1.30 (m, 12H, 6x CH₂ ester), 1.01 (s, 3H, 2'CCH₃).

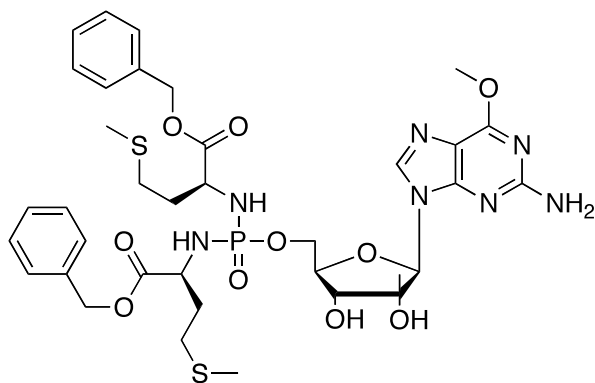
¹³C NMR (126 MHz, MeOD) δ 174.30, 174.21 (2d, ³J_{C-C-N-P} = 5.3 Hz, 2x C=O), 162.66 (C6), 161.987 (C2), 154.19 (C4), 139.34 (C8), 115.05 (C5), 93.33 (C1'), 82.61 (d, ³J_{C-C-O-P} = 6.5 Hz, C4'), 80.03 (C2'), 75.07 (OCH ester), 75.02 (OCH ester), 74.84 (C3'), 66.74 (d, ²J_{C-O-P} = 5.3 Hz, C5'), 54.87 (CHCH₂CH₂SCH₃), 54.79 (CHCH₂CH₂SCH₃), 54.41 (6OCH₃), 34.67 (d, ³J_{C-C-N-P} = 2.6 Hz, CHCH₂CH₂SCH₃), 34.62 (d, ³J_{C-C-N-P} = 2.6 Hz, CHCH₂CH₂SCH₃), 32.53 (CH₂ ester), 30.95 (CHCH₂CH₂SCH₃), 30.87 (CHCH₂CH₂SCH₃), 26.47 (CH₂ ester), 26.44 (CH₂ ester), 24.71 (CH₂ ester), 24.68 (CH₂ ester), 20.33 (2'CCH₃), 15.29 (CHCH₂CH₂SCH₃), 15.23 (CHCH₂CH₂SCH₃).

HPLC (System 1) t_R = 21.60 min.

MS (ES+) m/z: 840.32 (M+Na⁺, 100%).

HRMS C₃₄H₅₇N₇O₁₀P₁S₂ calculated: 818.3346 found: 818.3359.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-bis(benzyloxy-L-methioninyl) phosphate (8.2t).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl₃ (0.09 ml, 0.96 mmol), Et₃N (0.13 ml, 0.96 mmol), L-methionine benzyl ester tosylate salt

(**3.2m**, 1.94 g, 4.82 mmol), Et₃N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using CHCl₃/MeOH (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2t** was obtained as a white foam (130 mg, 16%).

^{31}P NMR (202 MHz, MeOD) δ 14.11.

^1H NMR (500 MHz, MeOD) δ 7.95 (s, 1H, H_8), 7.36-7.30 (2x OCH_2Ph) 5.97 (s, 1H, $\text{H}_{1'}$), 5.19-5.05 (m, 2x OCH_2Ph), 4.41-4.31 (m, 2H, $\text{H}_{5'}$ and $\text{H}_{3'}$), 4.19-4.17 (m, 1H, $\text{H}_{4'}$), 4.04 (s, 3H, 6OCH_3), 4.07-4.03 (m, 2H, 2x $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 2.51-2.39 (m, 4H, 2x $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.99 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.95 (s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.90-1.80 (m, 4H, 2x $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.00 (s, 3H, $2'\text{CCH}_3$).

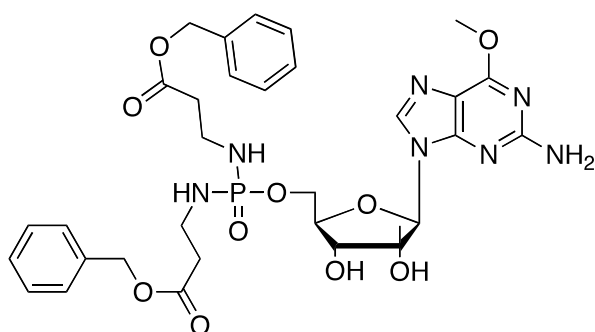
^{13}C NMR (126 MHz, MeOD) δ 173.78 (d, $^3J_{\text{C-C-N-P}} = 4.8$ Hz, C=O), 173.75 (d, $^3J_{\text{C-C-N-P}} = 4.8$ Hz, C=O), 161.91 (C6), 161.04 (C2), 153.73 (C4), 138.55 (C8), 136.39 (ipso OCH_2Ph), 136.35 (ipso OCH_2Ph), 128.77, 128.66, 128.63, 128.56, 128.53 (2x OCH_2Ph), 114.79 (C5), 92.37 (C1'), 81.59 (d, $^3J_{\text{C-C-O-P}} = 7.3$ Hz, C4'), 79.20 (C2'), 74.00 (C3'), 67.25 (d, $^2J_{\text{C-O-P}} = 6.3$ Hz, C5'), 65.81 (OCH_2Ph), 65.77 (OCH_2Ph), 53.87 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 53.72 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 53.42 (6OCH_3), 33.62 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 33.57 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 29.98 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 29.93 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 19.50 ($2'\text{CCH}_3$), 14.36 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 14.30 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$).

HPLC (System 1) $t_{\text{R}} = 19.05$ min.

MS (ES+) m/z : 856.25 ($\text{M} + \text{Na}^+$, 100%).

HRMS $\text{C}_{34}\text{H}_{57}\text{N}_7\text{O}_{10}\text{P}_1\text{S}_2$ calculated: 834.2720 found: 834.2692.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(benzoxy- β -alaninyl) phosphate (8.2t).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**5.4**, 300 mg, 0.96 mmol), POCl_3 (0.09 ml, 0.96 mmol), Et_3N (0.13 ml, 0.96 mmol), β -alanine benzyl ester tosylate salt

(1.69 g, 4.82 mmol), Et_3N (1.34 ml, 9.63 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography using $\text{CHCl}_3/\text{MeOH}$ (0 to 5%, gradient). Additional extraction from water using DCM was performed to remove remaining amino acid ester salt. The pure product **8.2t** was

obtained as a white foam (170 mg, 25%).

^{31}P NMR (202 MHz, MeOD) δ 17.45.

^1H NMR (500 MHz, MeOD) δ 8.00 (s, 1H, H₈), 7.32-7.25 (m, 10H, 2x OCH₂Ph), 6.00 (s, 1H, H_{1'}), 5.08-5.06 (m, 4H, 2x OCH₂Ph), 4.33-4.26 (m, 3H, H_{5'} and H_{3'}), 4.20- 4.17 (m, 1H, H_{4'}), 4.03 (s, 3H, 6OCH₃), 3.21-3.15 (m, 4H, 2x NHCH₂CH₂), 2.56 (t, J = 6.7 Hz, 2H, NHCH₂CH₂), 2.55 (t, J = 6.7 Hz, 2H, NHCH₂CH₂), 0.97 (s, 3H, 2'CCH₃).

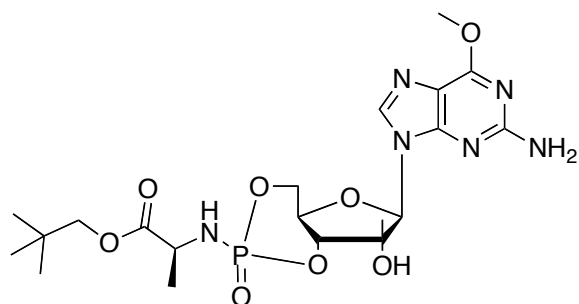
^{13}C NMR (126 MHz, MeOD) δ 173.42 (C=O), 162.72 (C6), 161.89 (C2), 154.63 (C4), 139.10 (C8), 137.52 (2x ipso OCH₂Ph), 129.87, 129.55, 129.21, 129.18 (2x OCH₂Ph), 115.53 (C5), 92.96 (C1'), 82.28 (d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C4'), 79.51 (C2'), 74.44 (C3'), 67.33 (2x OCH₂Ph), 65.48 (d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C5'), 54.29 (6OCH₃), 38.10 (d, $^3J_{\text{C-C-N-P}}$ = 5.0 Hz, NHCH₂CH₂), 37.99 (d, $^3J_{\text{C-C-N-P}}$ = 3.8 Hz, NHCH₂CH₂), 37.38 (NHCH₂CH₂), 37.34 (NHCH₂CH₂), 20.36 (2'CCH₃).

HPLC (System 1) t_{R} = 16.12 min.

MS (ES+) m/z : 736.32 (M+Na⁺, 100%).

HRMS C₃H₄₁N₇O₁₀P₁ calculated: 714.2653 found: 714.2623.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5',3'-*O*-(2,2-dimethylpropoxy-L-alaninyl) cyclic phosphate (8.6).



200 mg of **5.12I** (0.31 mmol) was dissolved in 5 ml of dry THF. L-alanine butyl ester hydrochloride salt (166 mg, 0.92 mmol) was added followed by the addition of 5 ml of DCM. The reaction mixture was

cooled down to -78 °C and DIPEA (0.32 ml, 1.84 mmol) was added, The reaction mixture was stirred for 20 min at -78 °C and after that time was allowed to warm to ambient temperature. When after 24 h, TLC analysis showed no product formation, reaction was heated up to 50 C and the reaction mixture was stirred for additional 24 h. After that time solvents were evaporated and the crude mixture was purified on

silica gel, using CHCl₃/MeOH (0 to 5%, gradient) as an eluent, to provide pure **8.6** as a white solid (22 mg, 14%).

³¹P NMR (202 MHz, MeOD) δ 7.56, 5.54.

¹H NMR (500 MHz, MeOD) δ 7.93, 7.92 (2s, 1H, H₈), 5.95, 5.94 (2s, 1H, H_{1'}), 4.71-4.64 (m, 1H, H_{3'}), 4.63-4.51 (m, 1H, H_{3'}, H_{5'a}), 4.35-4.30 (m, 1H, H_{5'b}), 4.08, 4.07 (2s, 3H, 6OCH₃), 4.05-4.00 (m, 1H, CHCH₃), 3.95, 3.91, 3.86, 3.77 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.51, 1.46 (2d, J = 7.5 Hz, 3H, CHCH₃), 1.15, 1.10 (2s, 3H, 2'CCH₃), 1.00, 0.99 (2s, 9H, OCH₂C(CH₃)₃).

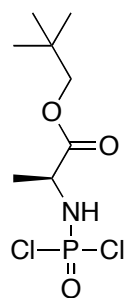
¹³C NMR (126 MHz, MeOD) δ 175.66, 175.02 (2d, ³J_{C-C-N-P} = 5.0 Hz, C=O), 162.82 (C6), 161.97, 161.90 (C2), 154.32 (C4), 140.30, 140.28 (C8), 115.96 (C5), 93.38, 93.22 (C1'), 82.85, 81.90 (C4'), 78.36 (d, ³J_{C-C-O-P} = 8.8 Hz, C2'), 78.21 (d, ³J_{C-C-O-P} = 7.6 Hz, C2'), 75.68, 75.47 (OCH₂C(CH₃)₃), 73.54, 72.67 (2d, ²J_{C-O-P} = 8.8 Hz, C3'), 70.73 (d, ²J_{C-O-P} = 8.8 Hz, C5'), 70.21 (d, ²J_{C-O-P} = 7.6 Hz, C5'), 54.21, 54.18 (6OCH₃), 50.85 (CHCH₃), 32.40 (OCH₂C(CH₃)₃), 26.81, 26.73 (OCH₂C(CH₃)₃), 20.63 (d, ³J_{C-C-N-P} = 5.0 Hz, CHCH₃), 20.45 (d, ³J_{C-C-N-P} = 6.3 Hz, CHCH₃), 19.94, 19.74 (2'CCH₃).

HPLC (System 1) t_R = 13.96 min.

MS (ES⁺) m/z: 515.20 (M+Na⁺, 100%).

HRMS calculated for C₂₀H₃₂N₆O₈PNa: 515.2019; found 515.1998.

Synthesis of 2,2-dimethylpropoxy-L-alaninyl phosphorodichloridate (**8.7a**).

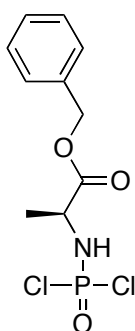


To a solution of L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 2.5 g, 7.54 mmol) and POCl₃ (0.69 ml, 7.54 mmol) in dry DCM (50 ml), Et₃N (1.92 ml, 15.08 mmol) was added at -78 °C. The reaction mixture was stirred at -78 °C for 1 h and after that time was allowed to warm to ambient temperature and was stirred for additional 1.5 h. After that time solvent was removed in vacuum and the crude was dissolved in diethyl ether (30 ml) and salts were filtered off. The solvent was evaporated and the product **8.7b** was obtained as clear oil (1.77 g, 83%).

³¹P NMR (202 MHz, CDCl₃) δ 13.21.

^1H NMR (500 MHz, CDCl_3) 4.96 (bs, 1H, NH), 4.29–4.16 (m, 1H, CHCH_3), 3.97, 3.95, 3.91, 3.88 (2AB, $J_{\text{AB}} = 10.5$ Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.56, 1.55 (2d, $J = 5.5$ Hz, 3H, CHCH_3), 0.98, 0.97 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

Synthesis of benzoxy-L-alaninyl phosphorodichloridate (**8.7b**).

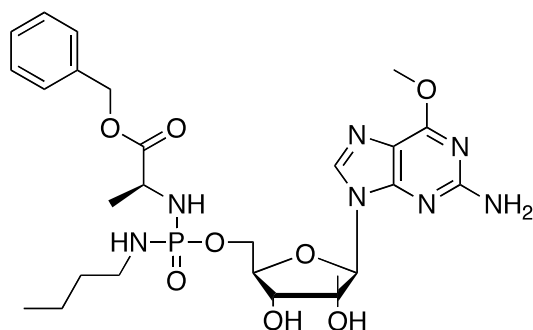


To a solution of L-alanine benzyl ester tosylate salt (2.5 g, 7.11 mmol) and POCl_3 (0.65 ml, 7.54 mmol) in dry DCM (50 ml), Et_3N (1.81 ml, 14.22 mmol) was added at -78°C . The reaction mixture was stirred at -78°C for 1 h and after that time was allowed to warm to ambient temperature and was stirred for additional 1.5 h. After that time solvent was removed in vacuum and the crude was dissolved in diethyl ether (30 ml) and salts were filtered off. The solvent was evaporated and the product **8.7b** was obtained as clear oil (1.68 g, 80%).

^{31}P NMR (202 MHz, CDCl_3) δ 12.77.

^1H NMR (500 MHz, CDCl_3) δ 7.44–7.37 (m, 5H, OCH_2Ph), 5.27 (s, 2H, OCH_2Ph), 4.95 (bs, 1H, NH), 4.27–4.21 (m, 1H, CHCH_3), 1.55 (d, $J = 7.0$ Hz, 3H, CHCH_3).

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-butylamine-(benzoxy-L-alaninyl) phosphate (**8.8a**).



Prepared according to the Standard Procedure 13c: In a first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 200 mg, 0.64 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with triethylamine (0.11 ml, 0.771 mmol) and

L-alaninyl benzyl ester phosphorodichloridate (**8.7b**, 380 mg, 1.29 mmole). Butylamine (0.32 ml, 3.21 mmole) and triethylamine (0.45 ml, 3.21 mmol) were added. After silica gel column chromatography, using $\text{CHCl}_3/\text{MeOH}$ as an eluent, the pure **8.8a** was obtained as an off white solid (43 mg, 11%).

^{31}P NMR (202 MHz, MeOD) δ 16.25, 16.09.

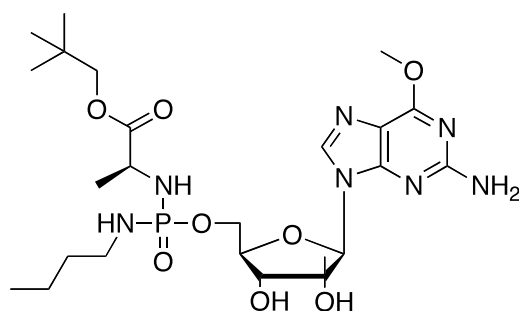
^1H NMR (500 MHz, MeOD) δ 7.99, 7.98 (2s, 1H, H_8), 5.99, 5.98 (2s, 1H, $\text{H}_{1'}$), 4.35-4.34 (m, 2H, $\text{H}_{5'}$), 4.27 (d, $J=9.0$ Hz, 1H, $\text{H}_{3'}$), 4.19- 4.17 (m, 1H, $\text{H}_{4'}$), 4.07 (s, 3H, 6OCH_3), 3.91- 3.79 (m, 1H, CHCH_3), 3.83, 3.70 (AB, $J_{\text{AB}}= 12.0$ Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.92- 2.87 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.47-1.43 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.37 (d, $J=7.5$ Hz, 3H, CHCH_3), 1.33- 1.29 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 0.99, 0.98 (2s, 3H, $2'\text{CCH}_3$), 0.94, 0.93 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 0.89 (t, $J= 7.5$ Hz, 3H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$).

HPLC (System 1) $t_{\text{R}} = 13.79$ min.

MS (EI+) m/z : 607.25 ($\text{M}+\text{H}^+$, 100%).

HRMS $\text{C}_{26}\text{H}_{38}\text{N}_7\text{O}_8\text{P}_1$ Calculated: 607.2520 found: 607.2504.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-butylamine-(2,2-dimethylpropyl-L-alaninyl) phosphate (8.8b).



Prepared according to the Standard Procedure 13c: In a first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 200 mg, 0.64 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with triethylamine (0.11 mL, 0.771

mmol) and L-alaninyl 2,2-dimethylpropyl ester phosphorodichloridate (**8.7a**, 355 mg, 1.29 mmole). Butylamine (0.32 mL, 3.21 mmole) and triethylamine (0.45 mL, 3.21 mmol) were added. After silica gel column chromatography, using $\text{CHCl}_3/\text{MeOH}$ as an eluent, the pure **8.8b** was obtained as an off white solid (11 mg, 3%).

^{31}P NMR (202 MHz, MeOD) δ 16.25, 16.09.

^1H NMR (500 MHz, MeOD) δ 7.99, 7.98 (2 s, 1H, H_8), 5.99, 5.98 (2s, 1H, $\text{H}_{1'}$), 4.35-4.34 (m, 2H, $\text{H}_{5'}$), 4.27 (d, $J= 9.0$ Hz, 1H, $\text{H}_{3'}$), 4.19- 4.17 (m, 1H, $\text{H}_{4'}$), 4.07 (s, 3H, 6OCH_3), 3.91- 3.79 (m, 1H, CHCH_3), 3.83, 3.70 (AB, $J_{\text{AB}}= 12.0$ Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 2.92- 2.87 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.47- 1.43 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.37 (d, $J= 7.5$ Hz, 3H, CHCH_3), 1.33- 1.29 (m, 2H,

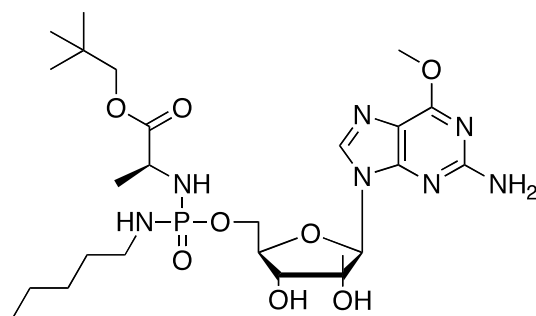
NHCH₂CH₂CH₂CH₃), 0.99, 0.98 (2 s, 3H, 2'CCH₃), 0.94, 0.93 (2s, 9H, OCH₂C(CH₃)₃), 0.89 (t, *J* = 7.5 Hz, 3H, NHCH₂CH₂CH₂CH₃).

HPLC (System 1) *t*_R = 14.71 min.

MS (EI+) *m/z*: 587.28 (M+H⁺, 100%).

HRMS C₂₄H₄₂N₇O₈P₁ Calculated: 587.2833 found: 587.2813.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-pentylamine-(2,2-dimethylpropyl-*L*-alaninyl) phosphate (8.8c).



Prepared according to the Standard Procedure 13c: In a first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 200 mg, 0.64 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with

triethylamine (0.11 mL, 0.771 mmol) and L-alaninyl 2,2-dimethylpropyl ester phosphorodichloridate (**8.7a**, 355 mg, 1.29 mmole). Pentylamine (0.56 mL, 3.21 mmole) and triethylamine (0.45 mL, 3.21 mmol) were added. After silica gel column chromatography, using CHCl₃/MeOH as an eluent, the pure **8.8c** was obtained as an off white solid (14 mg, 3%).

³¹P NMR (202 MHz, MeOD) δ 16.28, 16.12.

¹H NMR (500 MHz, MeOD) δ 8.02, 8.01 (2 s, 1H, H₈), 6.00, 5.99 (2 s, 1H, H_{1'}), 4.36-4.33 (m, 2H, H_{5'}), 4.29, 4.28 (2d, *J* = 9.0 Hz, 1H, H_{3'}), 4.20-4.17 (m, 1H, H_{4'}), 4.07 (s, 3H, 6OCH₃), 3.94-3.90 (m, 1H, CHCH₃), 3.87, 3.83, 3.75, 3.70 (2AB, *J*_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 2.90-2.85 (m, 2H, NHCH₂CH₂CH₂CH₂CH₃), 1.50-1.43 (m, 2H, NHCH₂CH₂CH₂CH₂CH₃), 1.39, 1.37 (2d, *J* = 6.0 Hz, 3H, CHCH₃), 1.31-1.21 (m, 4H, NHCH₂CH₂CH₂CH₂CH₃ and NH-CH₂CH₂CH₂CH₂CH₃), 0.99, 0.98 (2s, 3H, 2'CCH₃), 0.94, 0.93 (2s, 9H, OCH₂C(CH₃)₃), 0.87-0.85 (m, 3H, NHCH₂CH₂CH₂CH₂CH₃).

¹³C NMR (127 MHz, MeOH-*d*₄) δ 175.75, 175.68 (C=O), 162.72 (C6), 161.92 (C2), 154.61, 154.57 (C4), 139.25, 139.11 (C8), 115.43 (C5), 93.05, 92.94 (C1'), 82.41, 82.34 (2d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 80.08, 80.04 (C2'), 75.34, 75.31 (OCH₂C(CH₃)₃), 74.60, 74.37 (C3'), 65.90, 65.49 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.21 (6OCH₃), 51.27,

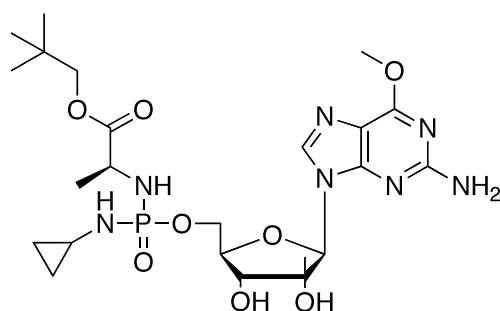
51.16 (CHCH₃), 42.06 (NHCH₂CH₂CH₂CH₂CH₃), 32.76 (d, ³J_{C-C-N-P}=5.0 Hz NHCH₂CH₂CH₂CH₂CH₃), 32.72 (d, ³J_{C-C-N-P}=6.3 Hz NHCH₂CH₂CH₂CH₂CH₃), 32.27 (OCH₂C(CH₃)₃), 30.11, 30.08 (NHCH₂CH₂CH₂CH₂CH₃), 26.74, 26.72 (OCH₂C(CH₃)₃), 23.46 (NHCH₂CH₂CH₂CH₂CH₃), 21.19 (d, ³J_{C-C-N-P}=6.3 Hz, CHCH₃), 21.96 (d, ³J_{C-C-N-P}=6.3 Hz, CHCH₃), 20.24 (2'CCH₃), 14.40 (NHCH₂CH₂CH₂CH₂CH₃).

HPLC (System 1) *t*_R = 17.11 min.

MS (EI+) *m/z*: 624.34 (MNa⁺, 100%).

HRMS C₂₅H₄₅N₇O₈P₁ Calculated: 602.3067 found: 602.3057.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-cyclopropylamine-(2,2-dimethylpropyl-L-alaninyl) phosphate (8.8d).



Prepared according to the Standard Procedure 13c: In a first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 200 mg, 0.64 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with triethylamine (0.11 mL, 0.771 mmol)

and L-alaninyl 2,2-dimethylpropyl ester phosphorodichloridate (**8.7a**, 355 mg, 1.29 mmole). Cyclopropylamine (0.33 mL, 3.21 mmole) and triethylamine (0.45 mL, 3.21 mmol) were added. After silica gel column chromatography, using CHCl₃/MeOH as an eluent, the pure **8.8d** was obtained as an off white solid (14 mg, 3%).

³¹P NMR (202 MHz, MeOD) δ 16.03, 15.77.

¹H NMR (500 MHz, MeOD) δ 8.04, 8.03 (2s, 1H, H₈), 6.01, 5.99 (2s, 1H, H_{1'}), 4.42-4.31 (m, 2H, H_{5'}), 4.29-4.25 (m, 1H, H_{3'}), 4.20-4.17 (m, 1H, H_{4'}), 4.08, 4.07 (2s, 3H, 6OCH₃), 3.97-3.92 (m, 1H, CHCH₃), 3.87, 3.82, 3.75, 3.68 (2AB, *J*_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 2.41-2.36 (m, 1H, NHCH cyclopropylamine), 1.39 (d, *J* = 6.5 Hz, 3H, CHCH₃), 1.38 (d, *J* = 6.5 Hz, 3H, CHCH₃), 0.99, 0.98 (2s, 3H, 2'CCH₃), 0.94, 0.92 (2s, 9H, OCH₂C(CH₃)₃ ester), 0.58-0.52 (m, 4H, 2x CH₂ cyclopropylamine).

¹³C NMR (127 MHz, MeOD) δ 175.70, 175.67 (C=O), 162.71 (C6), 161.99 (C2), 154.62, 154.56 (C4), 139.24, 139.00 (C8), 115.42, 115.33 (C5), 93.07, 92.82 (C1'),

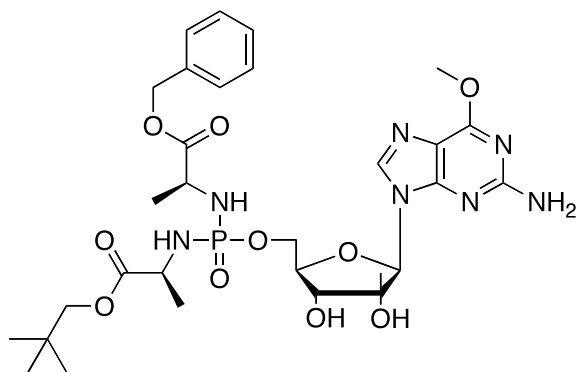
82.40, 82.24 (2d, $^3J_{C-C-O-P}=8.8$ Hz, C4'), 80.10, 80.04 (C2'), 75.35, 75.30 (OCH₂C(CH₃)₃). 74.56, 74.23 (C3'), 65.86, 65.23 (2d, $^2J_{C-O-P}=5.0$ Hz, C5'), 54.20 (6OCH₃), 51.30, 51.14 (CHCH₃), 32.26 (OCH₂C(CH₃)₃), 26.73, 26.70 (OCH₂C(CH₃)₃), 23.46 (NHCH cyclopropylamine), 21.23, 20.99 (2d, $^3J_{C-C-N-P}=6.3$ Hz, CHCH₃), 20.23 (2'CCH₃), 7.46, 7.26 (2x CH₂ cyclopropylamine).

HPLC (System 1) t_R = 13.17 min.

MS (EI+) m/z : 594.29 (MNa⁺, 100%).

HRMS C₂₃H₃₉N₇O₈P₁ Calculated: 572.2587 found: 572.2598.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-(2,2-dimethylpropoxy-L-alaninyl)-(benzoxy-L-alaninyl) phosphate (8.8e).



Prepared according to the Standard Procedure 13b: In the first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 250 mg, 0.80 mmol) in anhydrous tetrahydrofuran (5 ml) was allowed to react with triethylamine (0.11 ml, 0.80 mmol)

and phosphorus oxychloride (0.07 ml, 0.80 mmol). In the second step, L-alanine benzyl ester tosylate salt (282 mg, 0.80 mmol) and triethylamine (0.11 ml, 0.80 mmol) were added, followed by the addition of anhydrous dichloromethane (4 mL). In the third step, L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 1.33g, 4.02 mmol) and triethylamine (1.12 mL, 8.03 mmol) were added. After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), the pure compound **8.8e** was obtained as an off white solid (25 mg, 4%).

³¹P NMR (202 MHz, MeOD) 13.98, 13.94.

¹H NMR (500 MHz, MeOD) 7.97, 7.96 (2s, 1H, H₈), 7.36-7.30 (m, 5H, OCH₂Ph), 5.98, 5.97 (2s, 1H, H_{1'}), 5.18- 5.09 (m, 2H, OCH₂Ph), 4.39- 4.33 (m, 2H, H_{5'}), 4.28 (2d, $J= 8.00$ Hz, 1H, H_{3'}), 4.20- 4.16 (m, 1H, H_{4'}), 4.06, 4.05 (2s, 3H, 6OCH₃), 4.02- 3.94 (m, 2H, 2x CHCH₃), 3.84, 3.82, 3.72, 3.67 (2AB, $J_{AB}= 10.50$ Hz, 2H, CH₂C(CH₃)₃), 1.39- 1.32 (m, 6H, 2x CHCH₃), 0.97 (s, 3H, 2'CCH₃), 0.93, 0.91 (2s, 9H, CH₂C(CH₃)₃).

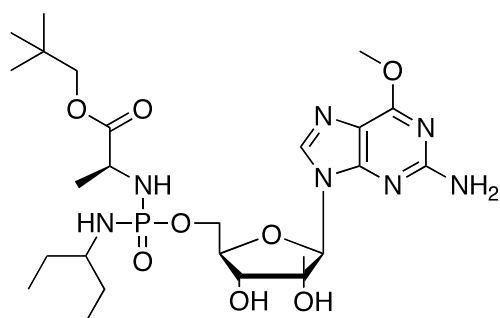
^{13}C NMR (126 MHz, MeOD) 175.54, 175.43, 175.39 (C=O), 162.73, 162.71 (C6), 161.93, 161.89 (C2), 154.57, 154.55 (C4), 139.32, 139.08 (C8), 137.39 (ipso OCH_2Ph), 129.55, 129.35, 129.25, 129.23, 129.20, 129.16, 128.27, 128.00 (OCH_2Ph), 116.19, 115.54 (C5), 93.34, 93.18 (C1'), 82.39, 82.33 (C4'), 80.01, 79.99 (C2'), 75.34, 75.04 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 74.84, 74.82 (C3'), 67.88, 67.85 (OCH_2Ph), 67.86 (d, $^2J_{\text{C-O-P}} = 3.75$ Hz, C5'), 66.36 (d, $^2J_{\text{C-O-P}} = 5.50$ Hz, C5'), 54.18, 54.01 (6OCH_3), 49.69, 49.64, 49.52, 49.46 (2x CHCH_3), 32.28, 32.25 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 26.74, 26.71 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 21.07, 20.90, 20.79, 20.66 (4d, $^3J_{\text{C-C-N-P}} = 6.25$ Hz, 2x CHCH_3), 20.39, 20.25 ($2'\text{CCH}_3$).

HPLC (System 1) $t_R = 16.11, 16.80$ min.

MS (ES+) m/z : 716.28 ($\text{M}+\text{Na}^+$, 100%).

HRMS $\text{C}_{30}\text{H}_{44}\text{N}_7\text{O}_{10}\text{P}_1$ calculated: 694.2966 found: 694.2956.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-diethylamine-(2,2-dimethylpropyl-L-alaninyl) phosphate (8.8f).



Prepared according to the Standard Procedure 13b: In the first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 300 mg, 0.96 mmol) in anhydrous tetrahydrofuran (5 ml) was allowed to react with triethylamine (0.16 ml, 0.96 mmol) and phosphorus oxychloride (0.11 ml, 0.96 mmol). In the second step, diethylamine (0.10 ml, 0.96 mmol) and triethylamine (0.13 ml, 0.96 mmol) were added. In the third step, L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 1.56g, 4.82 mmol) and triethylamine (1.34 mL, 9.64 mmol) were added followed by the addition of anhydrous dichloromethane (4 mL). After silica gel column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (0 to 5%, gradient), the pure compound **8.8f** was obtained as a white solid (15 mg, 3%).

^{31}P NMR (202 MHz, MeOD) δ 16.76, 16.68.

^1H NMR (500 MHz, MeOD) δ 7.99, 7.95 (2s, 1H, H_8), 5.99, 5.97 (2s, 1H, $\text{H}_{1'}$), 4.41-4.28 (m, 3H, $\text{H}_{5'}$ and $\text{H}_{3'}$), 4.21-4.17 (m, 1H, $\text{H}_{4'}$), 4.08 (s, 3H, 6OCH_3), 3.91-3.79 (m,

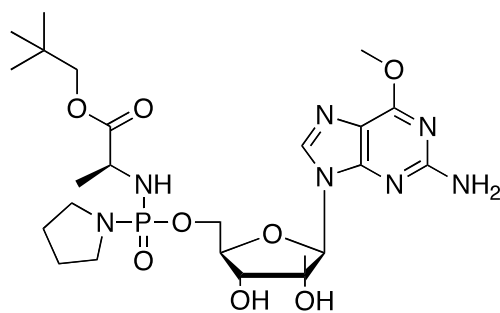
3H, CHCH₃ and OCH₂C(CH₃)₃), 3.18- 3.11 (m, 4H, NHCH(CH₂CH₃)CH₂CH₃), 2.40- 2.37 (m, 1H, NHCH(CH₂CH₃)CH₂CH₃), 1.40 (d, *J*= 7.5 Hz, 3H, CHCH₃), 1.11 (t, *J*= 7.0 Hz, 6H, NHCH(CH₂CH₃)CH₂CH₃), 0.99 (s, 3H, 2'CCH₃), 0.96, 0.93 (2s, 9H, OCH₂C(CH₃)₃).

HPLC (System 1) *t*_R = 11.68 min.

MS (EI+) *m/z*: 587.28 (MH⁺, 100%).

HRMS C₂₄H₄₂N₇O₈P₁ Calculated: 587.2833 found: 587.2813.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-pyrrolidine-(2,2-dimethylpropyl-L-alaninyl) phosphate (8.8g).



Prepared according to the Standard Procedure 13b: In the first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 250 mg, 0.80 mmol) in anhydrous tetrahydrofuran (5 ml) was allowed to react with triethylamine (0.11 ml, 0.80 mmol) and phosphorus oxychloride (0.07 ml, 0.80 mmol). In the second step, pyrrolidine (0.07 ml, 0.80 mmol) and triethylamine (0.11 ml, 0.80 mmol) were added. In the third step, L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 1.33 g, 4.02 mmol) and triethylamine (1.12 mL, 8.03 mmol) were added followed by the addition of anhydrous dichloromethane (4 mL). After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), the pure compound **8.8g** was obtained as a white solid (38 mg, 8%).

³¹P NMR (202 MHz, MeOD) δ 14.54, 14.42.

¹H NMR (500 MHz, MeOD) δ 8.01, 7.99 (2s, 1H, H₈), 6.00, 5.99 (2s, 1H, H_{1'}), 4.38- 4.35 (m, 2H, H_{5'}), 4.31- 4.28 (m, 1H, H_{3'}), 4.21- 4.19 (m, 1H, H_{4'}), 4.08, 4.07 (2s, 3H, 6OCH₃), 3.93- 3.91 (m, 1H, CHCH₃), 3.88- 3.71 (m, 2H, OCH₂C(CH₃)₃), 3.22-3.19 (m, 4H, 2x NCH₂ pyrrolidine), 1.87- 1.75 (m, 4H, 2x CH₂ pyrrolidine), 1.40, 1.37 (2d, *J*= 7.0 Hz, 3H, CHCH₃), 1.00, 0.99 (2s, 3H, 2'CCH₃), 0.95, 0.92 (2s, 9H, OCH₂C(CH₃)₃).

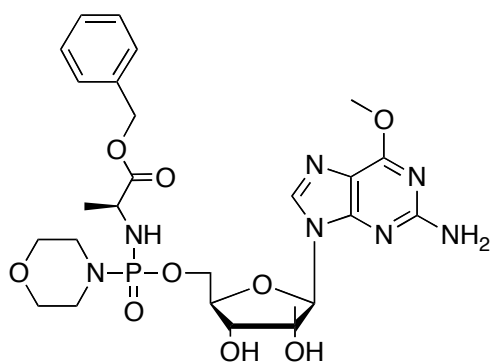
^{13}C NMR (126 MHz, MeOD) δ 175.71, 175.67 (C=O), 162.75, 162.73 (C6), 161.93 (C2), 154.56, 154.52 (C4), 139.16 (C8), 116.19, 115.57 (C5), 93.32, 93.16 (C1'), 82.44, 82.40 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 80.09, 80.03 (C2'), 75.34, 75.29 (OCH₂C(CH₃)₃), 74.64, 74.60 (C3'), 65.86 (d, $^2J_{\text{C-O-P}} = 8.80$ Hz, C5'), 65.80 (d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 54.24 (6OCH₃), 51.14, 51.02 (CHCH₃), 47.84 (d, $^2J_{\text{C-N-P}} = 3.8$ Hz, NCH₂ pyrrolidine), 47.84 (d, $^2J_{\text{C-N-P}} = 3.8$ Hz, NCH₂ pyrrolidine), 32.33 (OCH₂C(CH₃)₃), 27.34, 27.27 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, 2x CH₂ pyrrolidine), 26.78, 26.74 (OCH₂C(CH₃)₃), 21.20, 21.04 (2d, $^3J_{\text{C-C-N-P}} = 6.29$ Hz, CHCH₃), 20.32, 20.26 (2'CCH₃).

HPLC (System 1) $t_R = 13.41$ min.

MS (EI+) m/z : 585.27 (MH⁺, 100%).

HRMS C₂₄H₄₀N₇O₈P₁ Calculated: 585.2676 found: 585.2662.

Synthesis of 2-amino-6-*O*-methyl-9-(2-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-morpholine-(2,2-dimethylpropyl-L-alaninyl) phosphate (8.8h).



Prepared according to the Standard Procedure 13b: In the first step, a suspension of 6-*O*-methyl-2'-*C*-methylguanosine (**5.4**, 250 mg, 0.80 mmol) in anhydrous tetrahydrofuran (5 ml) was allowed to react with triethylamine (0.11 ml, 0.80 mmol) and phosphorus oxychloride

(0.07 ml, 0.80 mmol). In the second step, morpholine (0.08 ml, 0.80 mmol) and triethylamine (0.11 ml, 0.80 mmol) were added. In the third step, L-alanine benzyl ester tosylate salt (1.35 g, 4.02 mmol) and triethylamine (1.12 mL, 8.03 mmol) were added followed by the addition of anhydrous dichloromethane (4 mL). After silica gel column chromatography, using CHCl₃/MeOH (0 to 5%, gradient), the pure compound **8.8g** was obtained as a white solid (108 mg, 22%).

^{31}P NMR (202 MHz, MeOD) 14.65, 14.30.

^1H NMR (500 MHz, MeOD) 7.96, 7.95 (2s, 1H, H₈), 7.37-7.30 (m, 5H, OCH₂Ph), 5.98, 5.97 (2s, 1H, H_{1'}), 5.20-5.12 (m, 2H, OCH₂Ph), 4.42-4.33 (m, 3H, H_{5'} and H_{3'}),

4.22-4.18 (m, 1H, H_{4'}), 4.07, 4.06 (2s, 3H, 6OCH₃), 3.96-3.82 (m, 1H, CHCH₃), 3.52-3.79 (m, 4H, 2x O(CH₂)₂), 3.11-3.08 (m, 4H, 2x N(CH₂)₂), 1.38, 1.33 (2d, *J*=7.5 Hz, 3H, CHCH₃), 1.01, 1.00 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) 175.38, 175.34 (2d, ³*J*_{C-C-N-P}= 3.8 Hz, C=O), 162.79, 162.75 (C6), 161.91 (C2), 154.52, 154.47 (C4), 139.40, 139.31 (C8), 137.31 (ipso OCH₂Ph), 129.60, 129.57, 129.47, 129.38, 129.32 (OCH₂Ph), 115.71, 115.60 (C5), 93.46, 93.30 (C1'), 82.39, 82.33 (2d, ³*J*_{C-C-O-P}= 3.8 Hz, C4'), 80.04 (C2'), 74.98, 74.82 (C3'), 68.05 (d, ²*J*_{C-O-P}= 3.8 Hz, C5'), 67.97 (d, ²*J*_{C-O-P}= 5.0 Hz, C5'), 66.53, 66.49 (OCH₂Ph and O(CH₂)₂), 54.23 (6OCH₃), 51.24, 50.99 (CHCH₃), 45.74 (N(CH₂)₂), 20.79 (d, ³*J*_{C-C-N-P}= 5.0 Hz, CHCH₃), 20.55 (d, ³*J*_{C-C-N-P}= 6.3 Hz, CHCH₃), 20.29, 20.23 (2'CCH₃).

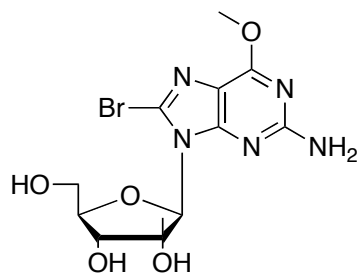
HPLC (System 1) *t*_R = 11.29, 11.60 min.

MS (ES+) *m/z*: 644.22 (M+Na⁺, 100%).

HRMS C₂₆H₃₇N₇O₉P₁ calculated: 622.2390 found: 622.2381.

12.8 Experimental section – Chapter Nine.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl)purine (9.1).



2-Amino-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl)purine (**5.4**, 1.50 g, 4.82 mmol) was suspended in dry MeOH (50 ml) and NBS (1.03 g, 4.82 mmol) was added. The mixture was stirred at room temperature for 4 h. After that time, solution was concentrated and the resulting solid was purified by silica gel chromatography using 1-4% (gradient) of MeOH in CHCl₃ to yield 1.65 g (88% yield) of the desired product **9.1**.

¹H NMR (500 MHz, MeOD) δ 6.04 (s, 1H, H_{1'}), 4.60 (d, *J* = 8.0 Hz, 1H, H_{3'}), 4.09-4.05 (m, 2H, H_{4'} and H_{5'a}), 4.04 (s, 3H, 6OCH₃), 3.98 (dd, *J* = 4.0 Hz, *J* = 12.5 Hz, 1H, H_{5'b}), 1.01 (s, 3H, 2'CCH₃).

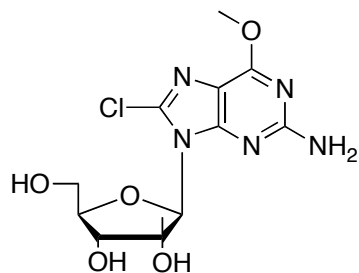
¹³C NMR (126 MHz, MeOD) δ 161.89 (C6), 161.18 (C2), 154.45 (C4), 125.57 (C8), 116.37 (C5), 96.70 (C1'), 84.40 (C4'), 80.38 (C2'), 74.34 (C3'), 62.27 (C5'), 54.66 (6OCH₃), 21.23 (2'CCH₃).

HPLC (System 1) *t_R* = 7.37 min.

MS (AP+) *m/z*: 390.04 (M+H⁺, 100%).

HRMS C₁₂H₁₇N₅O₅Br₁ Calculated: 390.0413 found: 390.0400.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl)purine (9.2).



2-Amino-6-methoxy-9-(2'-*C*-methyl-β-*D*-ribofuranosyl)purine (**5.4**, 250 mg, 0.80 mmol) was suspended in dry THF (10 ml) and NCS (110 mg, 0.80 mmol) was added. The mixture was warmed to 35 °C and stirred in the dark for overnight. After that time, solution was concentrated and the resulting solid was purified by silica gel chromatography using 1-4% (gradient) of MeOH in CHCl₃ to yield 235 mg (85% yield) of the desired product **9.2**.

^1H NMR (500 MHz, MeOD) δ 6.03 (s, 1H, $\text{H}_{1'}$), 4.58 (d, J = 8.0 Hz, 1H, $\text{H}_{3'}$), 4.09-4.04 (m, 2H, $\text{H}_{4'}$ and $\text{H}_{5'a}$), 4.05 (s, 3H, 6OCH_3), 3.97 (dd, J = 4.0 Hz, J = 11.0 Hz, 1H, $\text{H}_{5'b}$), 1.03 (s, 3H, $2'\text{CCH}_3$).

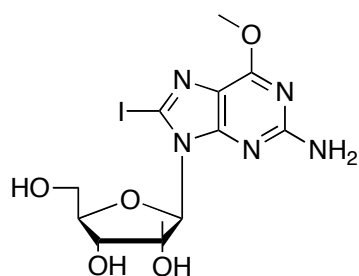
^{13}C NMR (126 MHz, MeOD) δ 162.02(C6), 161.30(C2), 154.35 (C4), 136.59 (C8), 114.52 (C5), 95.55 ($\text{C}_{1'}$), 84.29 ($\text{C}_{4'}$), 80.31 ($\text{C}_{2'}$), 74.42 ($\text{C}_{3'}$), 62.35 ($\text{C}_{5'}$), 54.55 (6OCH_3), 21.03 ($2'\text{CCH}_3$).

HPLC (System 1) t_R = 7.63 min.

MS (AP+) m/z : 346.09 ($\text{M}+\text{H}^+$, 100%).

HRMS $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_5\text{Cl}_1$ Calculated: 346.0918 found: 346.0901.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (9.3).



2-Amino-6-methoxy-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (**5.4**, 1.50 g, 4.82 mmol) was suspended in dry THF (40 ml) and NIS (1.10 g, 4.82 mmol) was added. The mixture was warmed to 35 °C and stirred in the dark for 3 days. After that time, solution was concentrated and the resulting solid was

purified by silica gel chromatography using 1-4% (gradient) of MeOH in CHCl_3 to yield 1.01 g (51% yield) of the desired product **9.3**.

^1H NMR (500 MHz, MeOD) δ 6.00 (s, 1H, $\text{H}_{1'}$), 4.64 (d, J = 9.0 Hz, 1H, $\text{H}_{3'}$), 4.07-3.98 (m, 3H, $\text{H}_{4'}$ and $\text{H}_{5'}$), 4.05 (s, 3H, 6OCH_3), 1.01 (s, 3H, $2'\text{CCH}_3$).

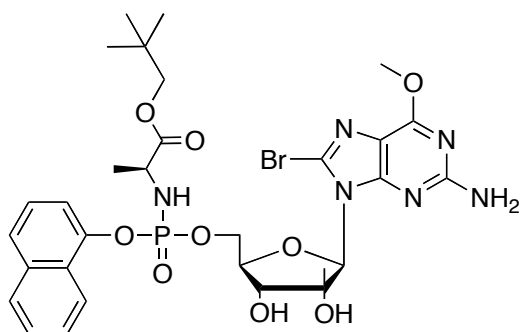
^{13}C NMR (126 MHz, MeOD) δ 161.70 (C6), 161.08 (C2), 154.28 (C4), 119.18 (C5), 99.27 (C8), 98.71 ($\text{C}_{1'}$), 84.39 ($\text{C}_{4'}$), 80.31 ($\text{C}_{2'}$), 74.52 ($\text{C}_{3'}$), 62.40 ($\text{C}_{5'}$), 54.41 (6OCH_3), 21.25 ($2'\text{CCH}_3$).

HPLC (System 1) t_R = 7.65 min.

MS (AP+) m/z : 438.03 ($\text{M}+\text{H}^+$, 100%).

HRMS $\text{C}_{12}\text{H}_{17}\text{N}_5\text{O}_5\text{I}_1$ Calculated: 438.0274 found: 438.0288.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (9.4a**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.1**, 160 mg, 0.41 mmol), *t*BuMgCl (0.81 ml, 0.81 mmol), and α -naphthyl-(2,2-dimethylpropoxy-L-

alaninyl) phosphorochloridate (**3.3e**, 270 mg, 0.81 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.4a** was obtained as an off white solid (70 mg, 23% yield).

³¹P NMR (202 MHz, MeOD) δ 4.15, 3.71.

¹H NMR (500 MHz, MeOD) δ 8.17-8.15, 8.01-8.00 (2m, 1H, H₈-Naph), 7.83-7.81, 7.75-7.73 (2m, 1H, H₅-Naph), 7.63, 7.64 (2d, *J* = 8.0 Hz, 1H, H₄-Naph), 7.50-7.41 (m, 3H, H₂, H₇, H₆-Naph), 7.36, 7.27 (2t, *J* = 8.0 Hz, 1H, H₃-Naph), 6.05, 6.04 (2s, 1H, H_{1'}), 4.86-4.82 (m, 2H, H_{3'} and H_{5'a}), 4.67-4.57 (m, 1H, H_{5'b}), 4.35-4.27 (m, 1H, H_{4'}), 4.08-4.01 (m, 1H, CHCH₃), 4.00, 3.96 (2s, 3H, 6OCH₃), 3.75, 3.72, 3.64, 3.58 (2AB, *J*_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.33, 1.27 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.05, 1.04 (2s, 3H, 2'CCH₃), 0.84, 0.82 (2s, 9H, OCH₂C(CH₃)₃).

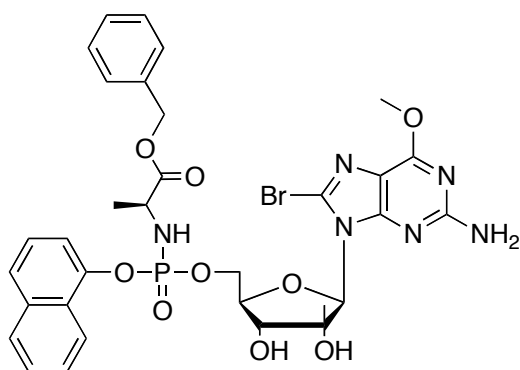
¹³C NMR (126 MHz, MeOD) δ 175.05, 174.77 (2d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.58, 161.49 (C6), 161.40, 161.27 (C2), 154.83, 154.75 (C4), 148.03, 147.96 (2d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 136.23, 136.09 (C10-Naph), 128.79, 128.63 (CH-Naph), 127.82, 127.71 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C9-Naph), 127.53, 127.38, 127.12, 126.44, 126.43, 125.82 (CH-Naph), 125.71 (C8), 125.59, 122.81, 122.69 (CH-Naph), 116.26, 116.23 (2d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 115.90, 115.87 (C5), 95.88 (C1'), 83.42 (d, ³*J*_{C-C-O-P} = 6.3 Hz, C4'), 83.24 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.19, 80.17 (C2'), 76.03, 75.93 (C3'), 75.34, 75.29 (OCH₂C(CH₃)₃), 69.51, 69.39 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.29, 54.27 (6OCH₃), 51.66, 51.60 (CHCH₃), 32.24 (OCH₂C(CH₃)₃), 26.72, 26.71 (OCH₂C(CH₃)₃), 20.82 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.69 (d, ³*J*_{C-C-N-P} = 7.5 Hz, CHCH₃), 20.56, 20.51 (2'CCH₃).

HPLC (System 1) *t*_R = 20.97, 21.77 min.

MS (ES⁺) *m/z*: 737.16 (M+H⁺, 100%).

HRMS C₃₀H₃₉N₆O₉P₁Br Calculated: 737.1700 found: 737.1683.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(benzoxy-L-alaninyl)] phosphate (9.4b).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.1**, 250 mg, 0.64 mmol), *t*BuMgCl (1.28 ml, 1.28 mmol), and α-naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3k**, 517 mg, 1.28

mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.4b** was obtained as an off white solid (63 mg, 13% yield).

³¹P NMR (202 MHz, MeOD) δ 4.17, 3.75.

¹H NMR (500 MHz, MeOD) δ 8.15, 7.99 (2d, 1H, *J* = 8.0 Hz, H₈-Naph), 7.83, 7.76 (2d, 1H, *J* = 8.0 Hz, H₅-Naph), 7.64, 7.54 (2d, *J* = 8.0 Hz, 1H, H₄-Naph), 7.50–7.41 (m, 3H, H₂, H₇, H₆-Naph), 7.37–7.32 (m, 1H, H₃-Naph), 7.27–7.22 (m, 5H, OCH₂Ph), 6.04, 6.02 (2s, 1H, H_{1'}), 5.20–4.99 (m, 2H, OCH₂Ph), 4.92–4.86 (m, 2H, H_{3'} and H_{5'a}), 4.63–4.56 (m, 1H, H_{5'b}), 4.32–4.24 (m, 1H, H_{4'}), 4.10–4.03 (m, 1H, CHCH₃), 3.97, 3.96 (2s, 3H, 6OCH₃), 1.33, 1.30 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.05, 1.04 (2s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.80, 174.63 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 161.57, 161.50 (C6), 161.38, 161.27 (C2), 154.82, 154.76 (C4), 148.03, 147.96 (2d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 137.41, 137.12 (ipso OCH₂Ph), 136.20, 136.07 (C10-Naph), 129.61, 129.51, 129.38, 129.34, 129.28, 129.21, 129.07, 128.78, 128.64, 128.29, 128.02, 127.90, 127.85, 127.80, 127.75, 127.68, 127.56, 127.39, 127.15, 126.45, 126.36 (CH-Naph and OCH₂Ph), 126.31, 125.71 (C8), 125.73, 125.62 (CH-Naph), 122.81, 122.72 (C5), 116.28 (d, ²*J*_{C-C-O-P} = 3.8 Hz, C2-Naph), 115.94 (d, ²*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 95.88 (C1'), 83.38, 83.20 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.19,

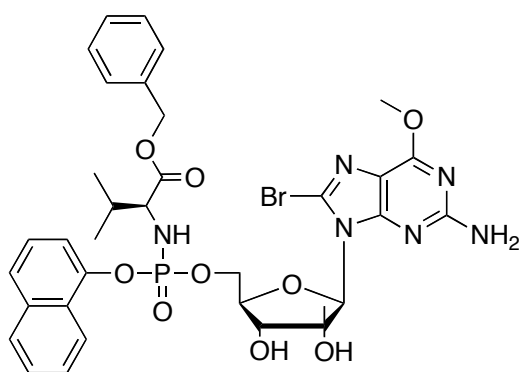
80.19 (C2'), 76.05, 76.00 (C3'), 69.43, 69.34 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 67.85, 67.77(OCH₂Ph), 54.30, 54.29 (6OCH₃), 51.67, 51.62 (CHCH₃), 20.56, 20.45 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃), 20.19 (2'CCH₃).

HPLC (System 1) $t_R = 19.76, 20.35$ min.

MS (ES+) m/z : 805.12 (M+H⁺, 100%).

HRMS C₃₂H₃₅N₆O₉P₁Br₁ Calculated: 805.1248 found: 805.1241.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(benzoxy-L-valinyl)] phosphate (9.4c).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.1**, 250 mg, 0.64 mmol), *t*BuMgCl (1.28 ml, 1.28 mmol), and α-naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3t**, 553 mg, 1.28

mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.4b** was obtained as an off white solid (131 mg, 26% yield).

³¹P NMR (202 MHz, MeOD) δ 4.94, 4.37.

¹H NMR (500 MHz, MeOD) δ 8.14, 7.98 (2d, 1H, $J = 7.5$ Hz, H₈-Naph), 7.83-7.80, 7.73-7.72 (2m, 1H, H₅-Naph), 7.63, 7.52 (2d, $J = 7.5$ Hz, 1H, H₄-Naph), 7.50-7.40 (m, 3H, H₂, H₇, H₆-Naph), 7.34-7.26 (m, 1H, H₃-Naph), 7.25-7.21 (m, 5H, OCH₂Ph), 6.04, 6.02 (2s, 1H, H_{1'}), 4.97-4.88 (m, 2H, OCH₂Ph), 4.85-4.78 (m, 2H, H₃ and H_{5'a}), 4.63-4.59 (m, 1H, H_{5'b}), 4.33-4.27 (m, 1H, H_{4'}), 3.97, 3.95 (2s, 3H, 6OCH₃), 3.80-3.72 (m, 1H, CHCH(CH₃)₂), 2.00-1.1.94, 1.91-1.84 (2m, 1H, CHCH(CH₃)₂), 1.06, 1.03 (2s, 3H, 2'CCH₃), 0.79, 0.73 (2d, $J = 6.5$ Hz, 3H, CHCH(CH₃)₂), 0.77, 0.72 (2d, $J = 6.0$ Hz, 3H, CHCH(CH₃)₂).

¹³C NMR (126 MHz, MeOD) δ 173.98 (d, $^3J_{C-C-N-P} = 3.7$ Hz, C=O), 173.81 (d, $^3J_{C-C-N-P} = 2.5$ Hz, C=O), 161.58, 161.48 (C6), 161.38, 161.24 (C2), 154.81, 154.74 (C4), 148.04, 147.97 (2d, $^2J_{C-O-P} = 7.5$ Hz, ipso Naph), 136.97 (ipso OCH₂Ph), 136.20,

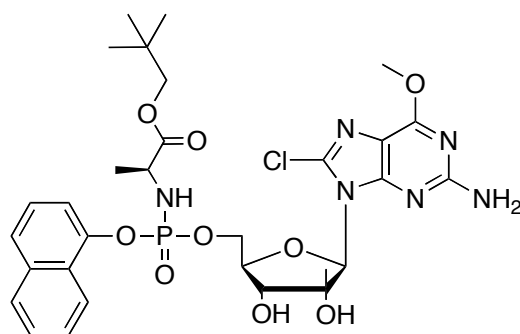
136.04 (C10-Naph), 129.63, 129.50, 129.44, 129.40, 129.31, 129.29, 128.79, 128.62, 127.90, 127.85, 127.77, 127.69, 127.54, 127.40, 127.10, 126.47 (CH-Naph and OCH₂Ph), 125.86, 125.80 (C8), 125.72, 125.59, 122.86, 122.75 (CH-Naph), 116.30, 116.28 (C5), 116.18 (d, $^2J_{C-C-O-P} = 3.8$ Hz, C2-Naph), 115.91 (d, $^2J_{C-C-O-P} = 2.5$ Hz, C2-Naph), 95.87, 95.83 (C1'), 83.53, 83.27 (2d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 80.19, 80.14 (C2'), 76.05, 75.94 (C3'), 69.64, 69.57 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 67.78 (OCH₂Ph), 61.88, 61.79 (CHCH(CH₃)₂), 54.35, 54.33 (6OCH₃), 33.34, 33.20 (2d, $^3J_{C-C-N-P} = 7.5$ Hz, CHCH(CH₃)₂), 20.61, 20.54 (2'CCH₃), 19.48, 19.35, 18.31, 18.24 (CHCH(CH₃)₂).

HPLC (System 1) $t_R = 21.80, 22.32$ min.

MS (AP+) m/z : 785.17 (M+H⁺, 100%).

HRMS C₃₄H₃₉N₆O₉P₁Br₁ Calculated: 785.1700 found: 785.1720.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (9.5a**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.2**, 160 mg, 0.41 mmol), *t*BuMgCl (0.81 ml, 0.81 mmol), and α-naphthyl-(2,2-dimethylpropoxy-L-

alaninyl) phosphorochloridate (**3.3e**, 270 mg, 0.81 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.5a** was obtained as an off white solid (70 mg, 23% yield).

³¹P NMR (202 MHz, MeOD) δ 4.16, 3.74.

¹H NMR (500 MHz, MeOD) δ 8.17-8.16, 8.02-8.00 (2m, 1H, H₈-Naph), 7.86- 7.85, 7.78-7.76 (2m, 1H, H₅-Naph), 7.67, 7.56 (2d, $J = 8.0$ Hz, 1H, H₄-Naph), 7.53-7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.27 (m, 1H, H₃-Naph), 6.02, 6.01 (2s, 1H, H₁'), 4.88-4.80 (m, 2H, H_{3'} and H_{5'a}), 4.63- 4.53 (m, 1H, H_{5'b}), 4.32- 4.24 (m, 1H, H_{4'}), 4.08-4.02 (m, 1H, CHCH₃), 4.01, 3.98 (2s, 3H, 6OCH₃), 3.76, 3.73, 3.65, 3.59 (2AB, $J_{AB} =$

10.5 Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.33, 1.27 (2d, $J = 7.0$ Hz, 3H, CHCH_3), 1.06, 1.05 (2s, 3H, $2'\text{CCH}_3$), 0.86, 0.84 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

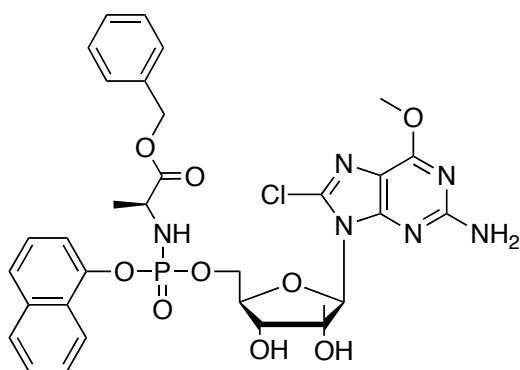
^{13}C NMR (126 MHz, MeOD) δ 174.76 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, $\text{C}=\text{O}$), 161.70, 161.61 (C6), 161.50, 161.38 (C2), 154.68, 154.61 (C4), 148.06, 147.96 (2d, $^2J_{\text{C-O-P}} = 7.5$ Hz, ipso Naph), 136.65, 136.58 (C8), 136.23, 136.09 (C10-Naph), 128.79, 128.64 (CH-Naph), 127.89, 127.78 (2d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.68, 127.53, 127.37, 127.12, 126.45, 126.34, 125.83, 125.60, 122.81, 122.70 (CH-Naph), 116.25 (d, $^3J_{\text{C-C-O-P}} = 3.8$ Hz, C2-Naph), 115.89 (d, $^3J_{\text{C-C-O-P}} = 2.5$ Hz, C2-Naph), 114.38 (C5), 94.67 (C1'), 83.39 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 83.20 (d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C4'), 80.13 (C2'), 75.99, 75.90 (C3'), 75.34, 75.30 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 69.45, 69.33 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 54.30, 54.28 (6OCH_3), 51.66, 51.60 (CHCH_3), 32.24, 32.23 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 26.72, 26.69 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 20.83, 20.70 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH_3), 20.48, 20.43 ($2'\text{CCH}_3$).

HPLC (System 1) $t_R = 20.89, 21.72$ min.

MS (ES+) m/z : 713.19 (MH^+ , 100%).

HRMS $\text{C}_{32}\text{H}_{35}\text{N}_6\text{O}_9\text{P}_1\text{Cl}_1$ Calculated: 713.1892 found: 713.1911.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-alaninyl)] phosphate (9.5b).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.2**, 250 mg, 0.64 mmol), *t*BuMgCl (1.28 ml, 1.28 mmol), and α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3k**, 517 mg, 1.28

mmol) in 5 mL of THF. After silica gel column chromatography using $\text{CHCl}_3/\text{MeOH}$ (0 to 3%, gradient), the pure compound **9.5b** was obtained as an off white solid (83 mg, 16% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.17, 3.79.

^1H NMR (500 MHz, MeOD) δ 8.17, 8.01 (2d, 1H, $J = 8.0$ Hz, $\text{H}_8\text{-Naph}$), 7.90, 7.83

(2d, 1H, $J = 8.0$ Hz, H₅- Naph), 7.71, 7.60 (2d, $J = 8.0$ Hz, 1H, H₄- Naph), 7.55–7.40 (m, 3H, H₂, H₇, H₆- Naph), 7.41–7.28 (m, 1H, H₃- Naph), 7.25–7.21 (m, 5H, OCH₂Ph), 6.02, 6.00 (2s, 1H, H₁'), 5.04–4.95 (m, 2H, OCH₂Ph), 4.90– 4.78 (m, 2H, H₃' and H₅'_a), 4.62– 4.56 (m, 1H, H₅'_b), 4.33– 4.25 (m, 1H, H₄''), 4.11–4.05 (m, 1H, CHCH₃), 3.95, 3.94 (2s, 3H, 6OCH₃), 1.37, 1.28 (2d, $J = 7.0$ Hz, 3H, CHCH₃), 1.04, 1.03 (2s, 3H, 2'CCH₃).

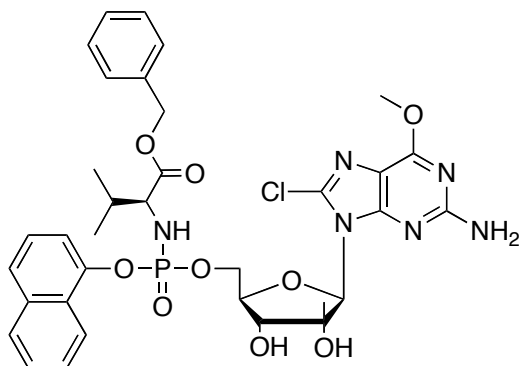
¹³C NMR (126 MHz, MeOD) δ 174.84, 174.63 (2d, $^3J_{C-C-N-P} = 5.0$ Hz, C=O), 161.69, 161.62 (C6), 161.45, 161.35 (C2), 154.68, 154.63 (C4), 148.01 (d, $^2J_{C-O-P} = 7.5$ Hz, ipso Naph), 147.95 (d, $^2J_{C-O-P} = 6.3$ Hz, ipso Naph), 137.08, 137.05 (ipso OCH₂Ph), 136.70, 136.62 (C8), 136.19, 136.07 (C10-Naph), 129.62, 129.53, 129.40, 129.35, 129.27, 129.23, 129.09, 128.81, 128.68 (CH-Naph and OCH₂Ph), 127.87, 127.77 (2d, $^3J_{C-C-O-P} = 3.8$ Hz, C9-Naph), 127.70, 127.58, 127.42, 127.18, 126.48, 126.39, 125.86, 125.67 (CH-Naph and OCH₂Ph), 122.81, 122.73 (C5), 116.31 (d, $^3J_{C-C-O-P} = 3.8$ Hz, C2-Naph), 115.97 (d, $^3J_{C-C-O-P} = 2.5$ Hz, C2-Naph), 94.65 (C1'), 83.35, 83.15 (2d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 80.17 (C2'), 76.02, 75.99 (C3'), 69.42, 69.30 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 67.89, 67.77 (OCH₂Ph), 54.37 (6OCH₃), 51.68, 51.63 (CHCH₃), 20.60, 20.55 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃), 20.34 (2'CCH₃).

HPLC (System 1) $t_R = 19.87, 20.48$ min.

MS (ES+) m/z : 713.19 (M+H⁺, 100%).

HRMS C₃₂H₃₅N₆O₉P₁Cl₁ Calculated: 713.1911 found: 713.1892.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-valinyl)] phosphate (9.5c).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.2**, 250 mg, 0.64 mmol), *t*BuMgCl (1.28 ml, 1.28 mmol), and α -naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3t**, 580 mg, 1.28

mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.5c** was obtained as an off white solid (90 mg, 17% yield).

³¹P NMR (202 MHz, MeOD) δ 4.93, 3.39.

¹H NMR (500 MHz, MeOD) δ 8.16, 8.02 (2d, 1H, *J* = 7.5 Hz, H₈-Naph), 7.85-7.82, 7.74-7.72 (m, 1H, H₅-Naph), 7.61, 7.54 (2d, *J* = 7.5 Hz, 1H, H₄-Naph), 7.50-7.40 (m, 3H, H₂, H₇, H₆-Naph), 7.36-7.30 (m, 1H, H₃-Naph), 7.26-7.21 (m, 5H, OCH₂Ph), 6.00, 5.99 (2s, 1H, H₁'), 4.98-4.87 (m, 2H, OCH₂Ph), 4.85-4.79 (m, 2H, H₃' and H₅'_a), 4.64-4.62 (m, 1H, H₅'_b), 4.37-4.30 (m, 1H, H₄'), 3.99, 3.96 (2s, 3H, 6OCH₃), 3.81-3.73 (m, 1H, CHCH(CH₃)₂), 2.07-1.86 (m, 1H, CHCH(CH₃)₂), 1.07, 1.05 (2s, 3H, 2'CCH₃), 0.82, 0.73 (2d, *J* = 6.5 Hz, 3H, CHCH(CH₃)₂), 0.79, 0.72 (2d, *J* = 6.0 Hz, 3H, CHCH(CH₃)₂).

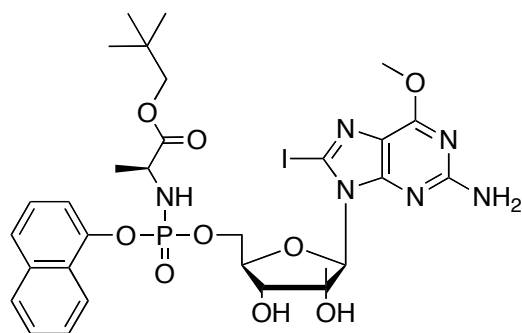
¹³C NMR (126 MHz, MeOD) δ 173.99 (d, ³*J*_{C-C-N-P} = 3.7 Hz, C=O), 173.84 (d, ³*J*_{C-C-N-P} = 2.5 Hz, C=O), 161.70, 161.61 (C6), 161.46, 161.32 (C2), 154.64, 154.59 (C4), 148.06, 147.97 (2d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 136.97, 136.93 (ipso OCH₂Ph), 136.71, 136.61 (C8), 136.20, 136.05 (C10-Naph), 129.50, 129.43, 129.41, 129.32, 129.31, 128.80, 128.63 (CH-Naph and OCH₂Ph), 127.87, 127.74, (2d, ³*J*_{C-C-O-P} = 3.8 Hz, C9-Naph), 127.55, 127.40, 127.12, 126.48, 126.34, 125.82, 125.61, 122.87, 122.76 (CH-Naph and OCH₂Ph), 122.87, 122.76 (C5), 116.17 (d, ²*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 115.90 (d, ²*J*_{C-C-O-P} = 3.7 Hz, C2-Naph), 94.63, 94.58 (C1'), 83.46, 83.19 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.14, 80.10 (C2'), 75.99, 75.89 (C3'), 69.57, 69.48 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 67.81 (OCH₂Ph), 61.87, 61.78 (CHCH(CH₃)₂), 54.39, 54.37 (6OCH₃), 33.35 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH(CH₃)₂), 33.20 (d, ³*J*_{C-C-N-P} = 7.5 Hz, CHCH(CH₃)₂), 20.85, 20.50 (2'CCH₃), 19.51, 19.38, 18.31, 18.25 (CHCH(CH₃)₂).

HPLC (System 1) t_R = 21.84, 22.31 min.

MS (ES+) m/z: 741.22 (M+H⁺, 100%).

HRMS C₃₄H₃₉N₆O₉P₁Cl₁ Calculated: 741.2205 found: 741.2185.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (9.6a**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 100 mg, 0.23 mmol), *t*BuMgCl (0.46 ml, 0.46 mmol), and α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 152 mg, 0.46 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6a** was obtained as an off white solid (46 mg, 27% yield).

³¹P NMR (202 MHz, MeOD) δ 4.18, 3.68.

¹H NMR (500 MHz, MeOD) δ 8.18-8.16, 7.99-7.97 (2m, 1H, H₈-Naph), 7.87- 7.85, 7.77-7.76 (2m, 1H, H₅-Naph), 7.67, 7.56 (2d, *J*= 8.0 Hz, 1H, H₄-Naph), 7.53 – 7.37 (m, 3H, H₂, H₇, H₆-Naph), 7.34-7.26 (m, 1H, H₃-Naph), 5.97, 5.96 (2s, 1H, H_{1'}), 4.89-4.82 (m, 2H, H_{3'} and H_{5'a}), 4.62- 4.53 (m, 1H, H_{5'b}), 4.32- 4.23 (m, 1H, H_{4'}), 4.08–3.99 (m, 1H, CHCH₃), 4.01, 3.97 (2s, 3H, 6OCH₃), 3.75, 3.72, 3.64, 3.58 (2AB, *J*_{AB}= 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.32, 1.25 (2d, *J*= 7.0 Hz, 3H, CHCH₃), 1.03, 1.02 (2s, 3H, 2'CCH₃), 0.87, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

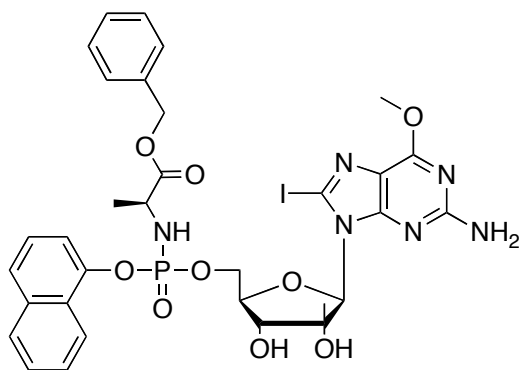
¹³C NMR (126 MHz, MeOD) δ 175.04 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 174.77 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.35, 161.27 (C6), 161.20, 161.06 (C2), 154.53, 154.45 (C4), 148.04, 147.97 (2d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 136.23, 136.09 (C10-Naph), 128.78, 128.62 (CH-Naph), 127.89, 127.77 (2d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.68, 127.53, 127.38, 127.14, 126.46, 126.33, 125.82, 125.59, 122.83, 122.68 (CH-Naph), 119.16, 119.12 (C5), 115.89, 115.86 (C2-Naph), 99.72 (C8), 98.04 (C1'), 83.36, 83.21 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.24, 80.21 (C2'), 76.12, 76.02 (C3'), 75.34, 75.28 (OCH₂C(CH₃)₃), 69.60, 69.44 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.22, 54.20 (6OCH₃), 51.66, 51.60 (CHCH₃), 32.25 (OCH₂C(CH₃)₃), 26.73 (OCH₂C(CH₃)₃), 20.81 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.70 (d, ³*J*_{C-C-N-P} = 5.0 Hz, CHCH₃), 20.66 (2'CCH₃).

HPLC (System 1) *t*_R = 20.23, 21.23 min.

MS (ES⁺) *m/z*: 785.15 (MH⁺, 100%).

HRMS $C_{30}H_{39}N_6O_9P_1I_1$ Calculated: 785.1546 found: 785.1561.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-alaninyl)] phosphate (9.6b**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α -naphthyl-(benzoxy-L-alaninyl) phosphorochloridate (**3.3k**, 550 mg, 1.37

mmol) in 5 mL of THF. After silica gel column chromatography using $CHCl_3/MeOH$ (0 to 3%, gradient), the pure compound **9.6b** was obtained as an off white solid (70 mg, 13% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.17, 3.69.

1H NMR (500 MHz, MeOD) δ 8.15, 7.98 (2d, 1H, $J = 8.0$ Hz, H_8 -Naph), 7.87, 7.78 (2d, 1H, $J = 8.0$ Hz, H_5 -Naph), 7.67, 7.56 (2d, $J = 8.0$ Hz, 1H, H_4 -Naph), 7.53–7.41 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.39–7.26 (m, 1H, H_3 -Naph), 7.28–7.23 (m, 5H, OCH_2Ph), 5.97, 5.96 (2s, 1H, $H_{1'}$), 5.00–4.91 (m, 2H, OCH_2Ph), 4.90–4.78 (m, 2H, $H_{3'}$ and $H_{5'a}$), 4.60–4.54 (m, 1H, $H_{5'b}$), 4.29–4.22 (m, 1H, $H_{4'}$), 4.08–4.02 (m, 1H, $CHCH_3$), 3.98, 3.97 (2s, 3H, $6OCH_3$), 1.30, 1.24 (2d, $J = 7.0$ Hz, 3H, $CHCH_3$), 1.03, 1.02 (2s, 3H, $2'CCH_3$).

^{13}C NMR (126 MHz, MeOD) δ 174.79, 174.60 (2d, $^3J_{C-C-N-P} = 5.0$ Hz, $C=O$), 161.34, 161.27 (C_6), 161.20, 161.07 (C_2), 154.50, 154.44 (C_4), 148.01, 147.95 (2d, $^2J_{C-O-P} = 7.5$ Hz, ipso Naph), 137.15, 137.12 (ipso OCH_2Ph), 136.21, 136.07 (C_{10} -Naph), 129.50, 129.36, 129.33, 129.28, 129.19, 129.09, 129.07, 128.75, 128.60, 128.27, 128.01, 127.91, 127.86, 127.80, 127.75, 127.65, 127.53, 127.37, 127.13, 126.43, 126.31, 125.80, 125.58, 122.82, 122.70 (CH -Naph and OCH_2Ph), 119.14, 119.09 (C_5), 116.28 (d, $^2J_{C-C-O-P} = 3.8$ Hz, C_2 -Naph), 115.90 (d, $^2J_{C-C-O-P} = 2.5$ Hz, C_2 -Naph), 99.75, 99.72 (C_8), 98.04 ($C_{1'}$), 83.33, 83.16 (2d, $^3J_{C-C-O-P} = 7.5$ Hz, $C_{4'}$), 80.22, 80.19 ($C_{2'}$), 76.09, 76.04 ($C_{3'}$), 69.52, 69.40 (2d, $^2J_{C-O-P} = 5.0$ Hz, $C_{5'}$),

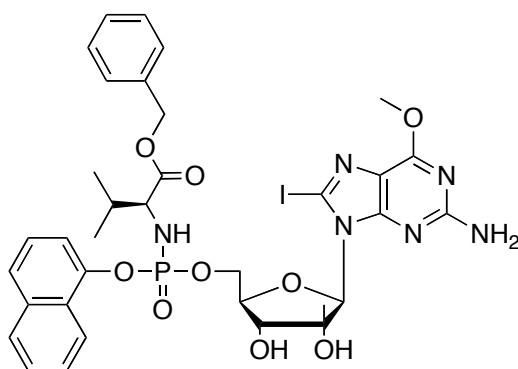
67.82, 67.76 (OCH₂Ph), 54.18, 54.16 (6OCH₃), 51.64, 51.61 (CHCH₃), 20.66, 20.45 (2d, ³J_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.10, 20.05 (2'CCH₃).

HPLC (System 1) t_R = 19.76, 20.35 min.

MS (ES+) m/z: 805.12 (MH⁺, 100%).

HRMS C₃₂H₃₅N₆O₉P₁I₁ Calculated: 805.1248 found: 805.1241.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(benzoxy-L-valinyl)] phosphate (9.6c).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(benzoxy-L-valinyl) phosphorochloridate (**3.3t**, 592 mg, 1.37

mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6c** was obtained as an off white solid (129 mg, 23% yield).

³¹P NMR (202 MHz, MeOD) δ 4.97, 4.37.

¹H NMR (500 MHz, MeOD) δ 8.14, 7.98 (2d, 1H, *J* = 7.5 Hz, H₈-Naph), 7.82-7.80, 7.73-7.71 (2m, 1H, H₅-Naph), 7.62, 7.51 (2d, *J* = 7.5 Hz, 1H, H₄-Naph), 7.48-7.38 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.29 (m, 1H, H₃-Naph), 7.24-7.19 (m, 5H, OCH₂Ph), 6.01, 5.99 (2s, 1H, H_{1'}), 4.97-4.88 (m, 2H, OCH₂Ph), 4.85-4.79 (m, 2H, H_{3'} and H_{5'a}), 4.64-4.61 (m, 1H, H_{5'b}), 4.33-4.28 (m, 1H, H_{4'}), 3.97, 3.94 (2s, 3H, 6OCH₃), 3.80-3.72 (m, 1H, CHCH(CH₃)₂), 2.04-1.83 (m, 1H, CHCH(CH₃)₂), 1.04, 1.02 (2s, 3H, 2'CCH₃), 0.79, 0.70 (2d, *J* = 6.5 Hz, 3H, CHCH(CH₃)₂), 0.77, 0.74 (2d, *J* = 6.0 Hz, 3H, CHCH(CH₃)₂).

¹³C NMR (126 MHz, MeOD) δ 174.02 (d, ³J_{C-C-N-P} = 3.7 Hz, C=O), 173.84 (d, ³J_{C-C-N-P} = 2.5 Hz, C=O), 161.38, 161.28 (C6), 161.20, 161.16 (C2), 154.53, 154.47 (C4), 148.04, 147.96 (2d, ²J_{C-O-P} = 7.5 Hz, ipso Naph), 136.97, 136.93 (ipso OCH₂Ph), 136.18, 136.03 (C10-Naph), 129.86, 129.73, 129.67, 129.64, 129.62, 129.54, 129.48, 129.43, 129.35, 128.83, 128.66, 127.90, 127.85, 127.73, 127.59, 127.45, 127.18,

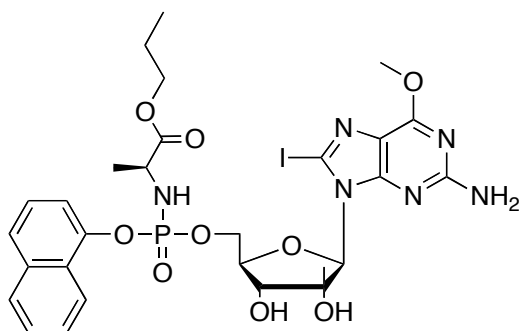
126.52, 126.37, 125.85, 125.64, 122.89, 122.76 (CH-Naph and OCH₂Ph), 119.27, 119.24 (C5), 116.22 (d, $^2J_{C-C-O-P}$ = 3.8 Hz, C2-Naph), 115.95 (d, $^2J_{C-C-O-P}$ = 2.5 Hz, C2-Naph), 99.83, 99.66 (C8), 97.99 (C1'), 83.50, 83.24 (2d, $^3J_{C-C-O-P}$ = 7.5 Hz, C4'), 80.30, 80.24 (C2'), 76.16, 76.04 (C3'), 69.72, 69.40 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 67.84, 67.76 (OCH₂Ph), 61.85, 61.76 (CHCH(CH₃)₂), 54.39, 54.37 (6OCH₃), 33.36 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH(CH₃)₂), 33.23 (d, $^3J_{C-C-N-P}$ = 7.5 Hz, CHCH(CH₃)₂), 20.90, 20.81 (2'CCH₃), 19.42, 19.37, 18.36, 18.31 (CHCH(CH₃)₂)

HPLC (System 1) t_R = 21.65, 22.11 min.

MS (ES+) m/z : 833.15 (MH⁺, 100%).

HRMS C₃₄H₃₉N₆O₉P₁I₁ Calculated: 833.1561 found: 833.1540.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(*n*-propyl-L-alaninyl)] phosphate (9.6d).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(*n*-propoxy-L-alaninyl) phosphorochloridate (**3.3c**, 488 mg, 1.37

mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6d** was obtained as an off white solid (100 mg, 17% yield).

³¹P NMR (202 MHz, MeOD) δ 4.22, 3.37.

¹H NMR (500 MHz, MeOD) δ 8.17-8.15, 8.00-7.98 (2m, 1H, H₈-Naph), 7.84-7.83, 7.76-7.75 (2m, 1H, H₅-Naph), 7.64, 7.55 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.51-7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.33-7.26 (m, 1H, H₃-Naph), 5.99, 5.98 (2s, 1H, H_{1'}), 4.91-4.84 (m, 2H, H_{3'} and H_{5'a}), 4.64-4.56 (m, 1H, H_{5'b}), 4.32-4.26 (m, 1H, H_{4'}), 4.04-3.97 (m, 1H, CHCH₃), 4.00, 3.96 (2s, 3H, 6OCH₃), 3.91-3.79 (m, 2H, OCH₂CH₂CH₃), 1.52-1.45 (m, 2H, OCH₂CH₂CH₃), 1.29, 1.23 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.03, 1.02 (2s, 3H, 2'CCH₃), 0.87, 0.84 (t, J = 7.0 Hz, 3H, OCH₂CH₂CH₃).

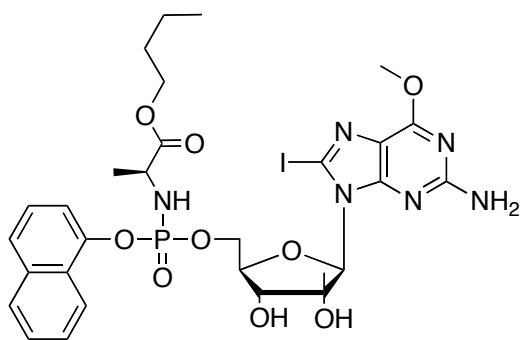
^{13}C NMR (126 MHz, MeOD) δ 175.12, 174.84 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, C=O), 161.37, 161.28 (C6), 161.18, 161.06 (C2), 154.53, 154.47 (C4), 148.04 (d, $^2J_{\text{C-O-P}} = 7.5$ Hz, ipso Naph), 147.98 (d, $^2J_{\text{C-O-P}} = 8.8$ Hz, ipso Naph), 136.20, 136.07 (C10-Naph), 128.79, 128.64 (CH-Naph), 127.89 (d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.76 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C9-Naph), 127.69, 127.56, 127.38, 127.14, 126.46, 126.35, 125.82, 125.59, 122.83, 122.69 (CH-Naph), 119.19, 119.14 (C5), 116.24, 115.87 (2d, $^3J_{\text{C-C-O-P}} = 2.5$ Hz, C2-Naph), 99.76, 99.65 (C8), 98.04 (C1'), 83.35 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 83.20 (d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C4'), 80.26, 80.25 (C2'), 76.15, 76.08 (C3'), 69.54, 69.41 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 67.86, 67.85 ($\text{OCH}_2\text{CH}_2\text{CH}_3$ ester), 54.29, 54.26 (6OCH_3), 51.61, 51.58 (CHCH_3), 22.90, 22.88 ($\text{OCH}_2\text{CH}_2\text{CH}_3$ ester), 20.78, 20.73 ($2'\text{CCH}_3$), 20.66, 20.61 (CHCH_3), 10.65 ($\text{OCH}_2\text{CH}_2\text{CH}_3$ ester).

HPLC (System 1) $t_{\text{R}} = 17.48, 18.29$ min.

MS (ES+) m/z : 757.12 (MH^+ , 100%).

HRMS $\text{C}_{28}\text{H}_{35}\text{N}_6\text{O}_9\text{P}_1\text{I}_1$ Calculated: 757.1248 found: 757.1229.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(*n*-butyl-L-alaninyl)] phosphate (9.6e**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α -naphthyl-(*n*-butoxy-L-alaninyl) phosphorochloridate (510 mg, 1.37 mmol)

in 5 mL of THF. After silica gel column chromatography using $\text{CHCl}_3/\text{MeOH}$ (0 to 3%, gradient), the pure compound **9.6e** was obtained as an off white solid (150 mg, 28% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.26, 3.68.

^1H NMR (500 MHz, MeOD) δ 8.17-8.15, 7.99-7.97 (2m, 1H, H_8 -Naph), 7.85-7.83, 7.76-7.75 (2m, 1H, H_5 -Naph), 7.64, 7.55 (2d, $J = 8.0$ Hz, 1H, H_4 -Naph), 7.51-7.35 (m, 3H, H_2 , H_7 , H_6 -Naph), 7.34-7.26 (m, 1H, H_3 -Naph), 5.99, 5.98 (2s, 1H, H_1),

4.91- 4.81 (m, 2H, H_{3'} and H_{5'a}), 4.65- 4.56 (m, 1H, H_{5'b}), 4.34- 4.26 (m, 1H, H_{4'}), 4.03-3.99 (m, 1H, CHCH₃), 4.00, 3.96 (2s, 3H, 6OCH₃), 3.92-3.83 (m, 2H, OCH₂CH₂CH₂CH₃), 1.46-1.39 (m, 2H, OCH₂CH₂CH₂CH₃), 1.30-1.23 (m, 5H, OCH₂CH₂CH₂CH₃ and CHCH₃), 1.03 (s, 3H, 2'CCH₃), 0.84, 0.83 (2t, *J* = 7.5 Hz, 3H, OCH₂CH₂CH₃).

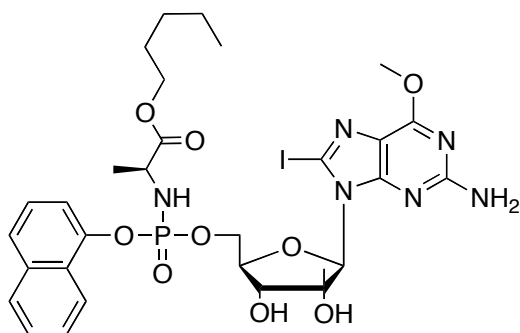
¹³C NMR (126 MHz, MeOD) δ 175.12 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 174.85 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.33, 161.25 (C6), 161.19, 161.06 (C2), 154.50, 154.44 (C4), 148.03 (d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 147.97 (d, ²*J*_{C-O-P} = 8.8 Hz, ipso Naph), 136.19, 136.05 (C10-Naph), 128.81, 128.65 (CH-Naph), 127.87 (d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.73 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C9-Naph), 127.57, 127.40, 127.16, 126.49, 126.37, 125.83, 125.59, 122.83, 122.69 (CH-Naph), 119.13, 119.09 (C5), 116.25, 115.83 (2d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 99.87, 99.76 (C8), 98.03 (C1'), 83.35, 83.20 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.25 (C2'), 76.14, 76.07 (C3'), 69.54, 69.40 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 66.12, 65.99 (OCH₂CH₂CH₂CH₃ ester), 54.31, 54.28 (6OCH₃), 51.60, 51.58 (CHCH₃), 31.65, 31.60 (OCH₂CH₂CH₂CH₃), 22.90, 22.88 (OCH₂CH₂CH₂CH₃), 20.80 (2'CCH₃), 20.74, 20.63 (2d, ³*J*_{C-C-N-P} = 7.5 Hz, CHCH₃), 14.14, 14.12 (OCH₂CH₂CH₂CH₃ ester).

HPLC (System 1) *t*_R = 18.92, 19.65 min.

MS (ES+) *m/z*: 771.14 (MH⁺, 100%).

HRMS C₂₉H₃₇N₆O₉P₁I₁ Calculated: 771.1404 found: 771.1400.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(*n*-propyl-L-alaninyl)] phosphate (9.6f).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(*n*-pentoxy-L-alaninyl) phosphorochloridate (500 mg, 1.37 mmol)

in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6f** was obtained as an off white solid (120 mg, 22% yield).

³¹P NMR (202 MHz, MeOD) δ 4.27, 3.68.

¹H NMR (500 MHz, MeOD) δ 8.17-8.15, 8.00-7.98 (2m, 1H, H₈-Naph), 7.85- 7.83, 7.76-7.75 (2m, 1H, H₅-Naph), 7.65, 7.55 (2d, *J* = 8.5 Hz, 1H, H₄-Naph) 7.51 – 7.35 (m, 3H, H₂, H₇, H₆-Naph), 7.34-7.26 (m, 1H, H₃-Naph), 5.99, 5.98 (2s, 1H, H₁'), 4.87-4.81 (m, 2H, H₃' and H₅'_a), 4.65-4.56 (m, 1H, H₅'_b), 4.34-4.26 (m, 1H, H₄'), 4.04–3.97 (m, 1H, CHCH₃), 4.00, 3.96 (2s, 3H, 6OCH₃), 3.92-3.81 (m, 2H, OCH₂CH₂CH₂CH₂CH₃), 1.47-1.41 (m, 2H, OCH₂CH₂CH₂CH₂CH₃), 1.29, 1.23 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.21-1.16 (m, 4H, OCH₂CH₂CH₂CH₂CH₃ and OCH₂CH₂CH₂CH₂CH₃), 1.03 (s, 3H, 2'CCH₃), 0.83, 0.82 (2t, *J* = 7.5 Hz, 3H, OCH₂CH₂CH₂CH₂CH₃).

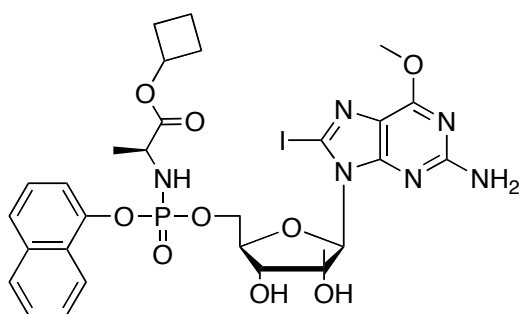
¹³C NMR (126 MHz, MeOD) δ 175.12, 174.86 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 161.33, 161.25 (C6), 161.20, 161.07 (C2), 154.51, 154.44 (C4), 148.03 (d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 147.97 (d, ²*J*_{C-O-P} = 8.8 Hz, ipso Naph), 136.19, 136.05 (C10-Naph), 128.81, 128.65 (CH-Naph), 127.87 (d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.74 (d, ³*J*_{C-C-O-P} = 8.8 Hz, C9-Naph), 127.57, 127.40, 127.15, 126.49, 126.37, 125.83, 125.59, 122.84, 122.70 (CH-Naph), 119.12, 119.08 (C5), 116.25, 115.83 (2d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 99.86, 99.77 (C8), 98.03 (C1'), 83.35, 83.20 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.25 (C2'), 76.14, 76.08 (C3'), 69.55 (d, ²*J*_{C-O-P} = 6.3 Hz, C5'), 69.40 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 66.39, 66.31 (OCH₂CH₂CH₂CH₂CH₃), 54.30, 54.27 (6OCH₃), 51.60, 51.58 (CHCH₃), 29.28, 29.23 (OCH₂CH₂CH₂CH₂CH₃), 29.06, 29.05 (OCH₂CH₂CH₂CH₂CH₃), 23.36, 23.34 (OCH₂CH₂CH₂CH₂CH₃), 20.79, 20.74 (2'CCH₃), 20.70, 20.62 (2d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 14.40 (OCH₂CH₂CH₂CH₂CH₃).

HPLC (System 1) t_R = 20.51, 21.31 min.

MS (ES+) m/z: 785.16 (MH⁺, 100%).

HRMS C₃₀H₃₉N₆O₉P₁I₁ Calculated: 785.1561 found: 785.1575.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(cyclobutoxy-L-alaninyl)] phosphate (9.6g**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α -naphthyl-(cyclobutoxy-L-alaninyl)

phosphorochloridate (510 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6g** was obtained as an off white solid (79 mg, 15% yield).

³¹P NMR (202 MHz, MeOD) δ 4.21, 3.63.

¹H NMR (500 MHz, MeOD) δ 8.18-8.16, 7.99-7.98 (2m, 1H, H₈-Naph), 7.87- 7.85, 7.78-7.77 (2m, 1H, H₅-Naph), 7.67, 7.57 (2d, *J*= 8.0 Hz, 1H, H₄-Naph), 7.53- 7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.27 (m, 1H, H₃-Naph), 5.98, 5.97 (2s, 1H, H_{1'}), 4.90- 4.80 (m, 2H, H_{3'} and H_{5'a}), 4.77-4.70 (m, 1H, OCH ester), 4.63- 4.54 (m, 1H, H_{5'b}), 4.32- 4.24 (m, 1H, H_{4'}), 4.01, 3.97 (2s, 3H, 6OCH₃), 3.96 – 3.92 (m, 1H, CHCH₃), 2.21-2.16 (m, 2H, 2x CH_{2a'} ester), 1.92-1.83 (m, 2H, 2x CH_{2a''} ester), 1.73- 1.66 (m, 1H, CH_{2b} ester), 1.61- 1.51 (m, 1H, CH_{2b} ester), 1.29, 1.23 (2d, *J*= 7.0 Hz, 3H, CHCH₃), 1.03, 1.02 (2s, 3H, 2'CCH₃).

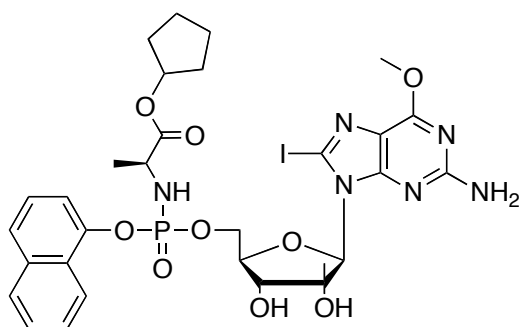
¹³C NMR (126 MHz, MeOD) δ 174.37 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 174.14 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.36, 161.27 (C6), 161.21, 161.07 (C2), 154.52, 154.45 (C4), 148.07, 148.01 (ipso Naph), 136.22, 136.08 (C10-Naph), 128.78, 128.62 (CH-Naph), 127.92, 128.87 (C9-Naph), 127.78, 127.68, 127.55, 127.37, 127.11, 126.44, 126.33, 125.80, 125.55, 122.83, 122.70 (CH-Naph), 119.15, 119.11 (C5), 116.23, 115.79 (2d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 99.76 (C8), 98.05 (C1'), 83.34 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 83.20 (d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 80.24, 80.21 (C2'), 76.12, 76.05 (C3'), 70.96, 70.95 (OCH ester), 69.53, 69.42 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.23, 54.19 (6OCH₃), 51.47, 51.44 (CHCH₃), 31.07, 30.92 (CH₂ ester), 20.72, 20.67 (2'CCH₃), 20.55, 20.44 (2d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 14.23, 14.21 (CH₂ ester).

HPLC (System 1) *t*_R = 17.80, 18.72 min.

MS (ES⁺) *m/z*: 769.12 (MH⁺, 100%).

HRMS C₂₉H₃₅N₆O₉P₁I₁ Calculated: 769.1248 found: 769.1222.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(cyclopentoxo-L-alaninyl)] phosphate (9.6h).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(cyclopropoxy-L-

alaninyl) phosphorochloridate (**3.3h**, 520 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6h** was obtained as an off white solid (80 mg, 15% yield).

³¹P NMR (202 MHz, MeOD) δ 4.25, 3.67.

¹H NMR (500 MHz, MeOD) δ 8.17-8.15, 7.98-7.97 (2m, 1H, H₈-Naph), 7.87- 7.85, 7.78-7.76 (2m, 1H, H₅-Naph), 7.67, 7.56 (2d, *J* = 8.5 Hz, 1H, H₄-Naph), 7.53– 7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.27 (m, 1H, H₃-Naph), 5.97, 5.96 (2s, 1H, H_{1'}), 4.97-4.92 (m, 1H, OCH ester), 4.90- 4.79 (m, 2H, H_{3'} and H_{5'a}), 4.62- 4.54 (m, 1H, H_{5'b}), 4.31- 4.23 (m, 1H, H_{4'}), 4.01, 3.97 (2s, 3H, 6OCH₃), 3.95– 3.89 (m, 1H, Hα Ala), 1.75- 1.70 (m, 2H, CH₂), 1.62- 1.59 (m, 6H, 3x CH₂ ester), 1.28, 1.20 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.03, 1.02 (2 x s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.88, 174.61 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 161.37, 161.28 (C6), 161.18, 161.04 (C2), 154.54, 154.47 (C4), 148.04 (d, ²*J*_{C-O-P} = 7.5 Hz, ipso Naph), 147.96 (d, ²*J*_{C-O-P} = 6.3 Hz, ipso Naph), 136.20, 136.06 (C10-Naph), 128.82, 128.66 (CH-Naph), 127.88, 128.74 (2d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.58, 127.40, 127.25, 127.16, 126.48, 126.36, 125.85, 125.62, 122.82, 122.68 (CH-Naph), 119.21, 119.17 (C5), 116.26 (d, ³*J*_{C-C-O-P} = 3.4 Hz, C2-Naph), 115.85 (d, ³*J*_{C-C-O-P} = 2.5 Hz, C2-Naph), 99.79, 99.66 (C8), 98.03, 97.84 (C1'), 83.36 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 83.22 (d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 80.28, 80.26 (C2'), 79.52, 79.48 (OCH ester), 76.19, 76.09 (C3'), 69.57 (d, ²*J*_{C-O-P} = 6.3 Hz, C5'), 69.22 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'),

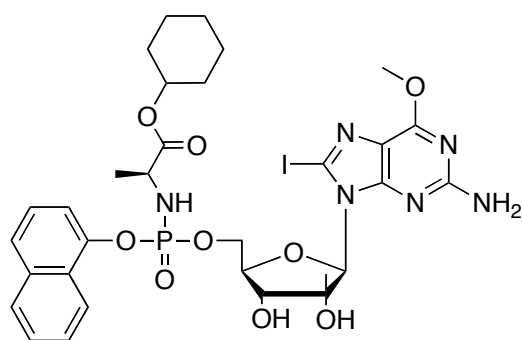
54.33, 54.30 (6OCH₃), 51.69, 51.63 (CHCH₃) 33.53, 33.43 (2x CH₂ ester), 24.64, 24.60 (2x CH₂ ester), 20.83, 20.79 (2'CCH₃), 20.68, 20.60 (2d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃).

HPLC (System 1) t_R = 18.99, 19.81 min.

MS (TOF ES+) m/z : 783.14 (MH⁺, 100%).

HRMS C₃₀H₃₇N₆O₉P₁I₁ Calculated: 783.1404 found: 783.1382.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(cyclohexoxy-L-alaninyl)] phosphate (9.6i).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(cyclohexoxy-L-alaninyl)

phosphorochloridate (**3.3i**, 550 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6i** was obtained as an off white solid (75 mg, 14% yield).

³¹P NMR (202 MHz, MeOD) δ 4.23, 3.66.

¹H NMR (500 MHz, MeOD) δ 8.17-8.15, 7.99-7.98 (2m, 1H, H₈-Naph), 7.86-7.84, 7.77-7.76 (2m, 1H, H₅-Naph), 7.66, 7.56 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.52-7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.27 (m, 1H, H₃-Naph), 5.98, 5.97 (2s, 1H, H_{1'}), 4.90-4.81 (m, 2H, H_{3'} and H_{5'a}), 4.63- 4.51 (m, 2H, H_{5'b} and OCH ester), 4.32- 4.24 (m, 1H, H_{4'}), 4.00, 3.97 (2s, 3H, 6OCH₃), 3.99- 3.91 (m, 1H, CHCH₃), 1.68- 1.63 (m, 4H, 2x CH₂ ester), 1.48- 1.47 (m, 1H, 1 of CH₂ ester), 1.34- 1.26 (m, 7H, 1 of CH₂ and 3x CH₂ ester), 1.29, 1.22 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.03 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOH-*d*₄) δ 174.50 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 174.24 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, C=O), 161.36, 161.27 (C6), 161.20, 161.06 (C2), 154.53, 154.46 (C4), 148.06 (d, $^2J_{C-O-P}$ = 7.5 Hz, ipso Naph), 147.99 (d, $^2J_{C-O-P}$ = 8.8 Hz, ipso Naph), 136.23, 136.09 (C10-Naph), 128.79, 128.62 (CH-Naph), 127.90, 128.76 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.54, 127.37, 127.11, 126.46, 126.33, 125.80, 125.55, 122.83,

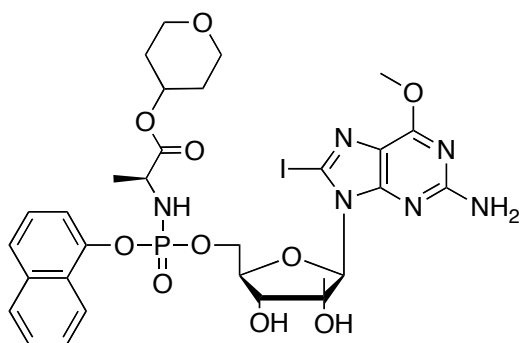
122.70 (CH-Naph), 119.16, 119.11 (C5), 116.23, 115.80 (2d, $^3J_{C-C-O-P} = 3.4$ Hz, C2-Naph), 99.73, 99.65 (C8), 98.04 (C1'), 83.37, 83.21 (2d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 80.24 (C2'), 76.12, 76.05 (C3'), 74.88 (OCH ester), 69.56, 69.43 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 54.24, 54.22 (6OCH₃), 51.74, 51.71 (CHCH₃) 32.32, 32.27 (2CH₂ ester), 26.37 (2CH₂ ester), 24.55 (CH₂ ester), 20.73, 20.69 (2'CCH₃), 20.72, 20.62 (2d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃).

HPLC (System 1) $t_R = 20.35, 21.16$ min.

MS (ES+) m/z : 819.14 (MNa⁺, 100%).

HRMS C₃₁H₃₈N₆O₉P₁I₁Na₁ Calculated: 819.1380 found: 819.1390.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(tetrahydropyroxyl-L-alaninyl)] phosphate (9.6j).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(tetrahydropyroxyl-L-

alaninyl) phosphorochloridate (**3.3i**, 550 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6j** was obtained as an off white solid (150 mg, 27% yield).

³¹P NMR (202 MHz, MeOD) δ 4.17, 3.61.

¹H NMR (500 MHz, MeOD) δ 8.18-8.16, 7.99-7.98 (2m, 1H, H₈-Naph), 7.86- 7.84, 7.77-7.76 (2m, 1H, H₅-Naph), 7.66, 7.56 (2d, $J = 8.0$ Hz, 1H, H₄-Naph), 7.52- 7.36 (m, 3H, H₂, H₇, H₆-Naph), 7.33-7.27 (m, 1H, H₃-Naph), 5.98, 5.97 (2s, 1H, H_{1'}), 4.90- 4.84 (m, 2H, H_{3'} and H_{5'a}), 4.77- 4.68 (m, 1H, OCH ester) 4.63- 4.51 (m, 1H, H_{5'b}), 4.32- 4.24 (m, 1H, H_{4'}), 4.03- 3.95 (m, 1H, CHCH₃), 4.01, 3.97 (2s, 3H, 6OCH₃), 3.80- 3.74 (m, 2H, OCH₂ ester), 3.47- 3.40 (m, 2H, OCH₂ ester), 1.77- 1.69 (m, 2H, OCHCH₂ ester), 1.52- 1.43 (m, 2H, OCHCH₂ ester), 1.30, 1.23 (2d, $J = 6.5$ Hz, 3H, CHCH₃), 1.03, 1.02 (s, 3H, 2'CCH₃).

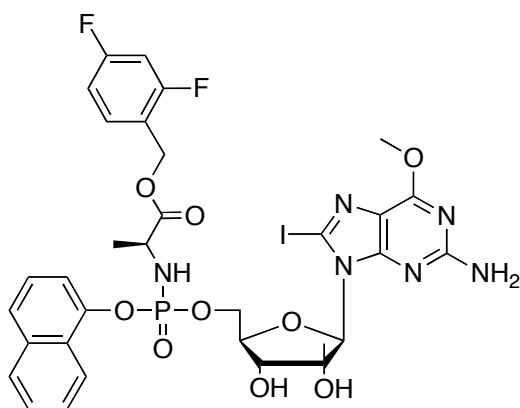
^{13}C NMR (126 MHz, MeOD) δ 174.30 (d, $^3J_{\text{C-C-N-P}} = 4.3$ Hz, C=O), 174.06 (d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, C=O), 161.37, 161.29 (C6), 161.21, 161.06 (C2), 154.55, 154.46 (C4), 148.03 (d, $^2J_{\text{C-O-P}} = 7.5$ Hz, ipso Naph), 147.97 (d, $^2J_{\text{C-O-P}} = 8.8$ Hz, ipso Naph), 136.23, 136.08 (C10-Naph), 128.82, 128.65 (CH-Naph), 127.89 (d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.74 (d, $^3J_{\text{C-C-O-P}} = 5.0$ Hz, C9-Naph), 127.58, 127.41, 127.15, 126.49, 126.37, 125.85, 125.59, 122.81, 122.67 (CH-Naph), 119.19, 119.13 (C5), 116.27, 115.82 (2d, $^3J_{\text{C-C-O-P}} = 3.4$ Hz, C2-Naph), 99.78, 99.66 (C8), 98.05 (C1'), 83.40 (d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C4'), 83.23 (d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 80.24, 80.22 (C2'), 76.13, 76.04 (C3'), 71.25, 71.22 (OCH ester), 69.59 (d, $^2J_{\text{C-O-P}} = 6.3$ Hz, C5'), 69.44 (d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 66.07, 66.01, 65.99 (O(CH₂)₂ ester), 54.28, 54.24 (6OCH₃), 51.70, 51.66 (CHCH₃) 32.50, 32.44, 32.41 (OCH(CH₂)₂ ester), 20.75, 20.71 (2'CCH₃), 20.63, 20.55 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH₃).

HPLC (Sstem 1) t_R = 14.91, 15.71 min.

MS (ES+) m/z : 799.13 (MH⁺, 100%).

HRMS C₃₀H₃₇N₆O₁₀P₁I₁ Calculated: 799.1354 found: 799.1349.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,4-di-fluorobenzoxy-L-alaninyl)] phosphate (9.6k).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α -naphthyl-(2,4-di-F-benzoxy-L-alaninyl) phosphorochloridate (603 mg, 1.37 mmol) in 5 mL of THF. After silica

gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6k** was obtained as an off white solid (120 mg, 20% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.16, 3.81.

^1H NMR (500 MHz, MeOD) δ 8.14- 8.12, 7.97- 7.96 (2m, 1H, H₈-Naph), 7.85- 7.83, 7.75- 7.74 (2m, 1H, H₅-Naph), 7.64, 7.54 (2d, $J = 8.0$ Hz, 1H, H₄-Naph), 7.50- 7.33 (m, 3H, H₂, H₇, H₆-Naph), 7.32-7.24 (m, 2H, H₃- Naph and OCH₂-2,4-F-Ph), 6.89-

6.78 (m, 2H, OCH₂-2,4-F-Ph), 5.96, 5.91 (2s, 1H, H_{1'}), 5.04-4.95 (m, 2H, OCH₂-2,4-F-Ph), 4.85- 4.76 (m, 2H, H_{3'} and H_{5'a}), 4.64- 4.55 (m, 1H, H_{5'b}), 4.28- 4.22 (m, 1H, H_{4'}), 4.08 – 4.02 (m, 1H, CHCH₃), 4.00, 3.99 (2s, 3H, 6OCH₃), 1.30, 1.24 (2d, $J = 7.0$ Hz, 3H, CHCH₃), 1.01 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 174.62, 174.43 (2d, $^3J_{C-C-N-P} = 5.0$ Hz, C=O), 159.50 (d, $J_{C-F} = 248.5$ Hz, OCH₂-2,4-F-Ph), 164.42 (d, $J_{C-F} = 243.7$ Hz, OCH₂-2,4-F-Ph), 162.46 (d, $J_{C-F} = 246.7$ Hz, OCH₂-2,4-F-Ph), 162.33 (d, $J_{C-F} = 251.2$ Hz, OCH₂-2,4-F-Ph), 161.46, 161.42 (C6), 161.29, 161.25 (C2), 154.50, 154.44 (C4), 148.00, 147.90 (2d, $^2J_{C-O-P} = 7.5$ Hz, ipso Naph), 136.19, 136.01 (C10-Naph), 133.08, 133.05 (2d, $^3J_{C-F} = 7.1$ Hz, OCH₂-2,4-F-Ph), 128.78, 128.61 (CH-Naph), 127.85 (d, $^3J_{C-C-O-P} = 6.3$ Hz, C9-Naph), 127.72 (d, $^3J_{C-C-O-P} = 5.0$ Hz, C9-Naph), 127.55, 127.38, 127.11, 126.43, 126.32, 125.85, 125.64, 122.78, 122.68 (CH-Naph), 120.51-120.43 (m, ipso OCH₂-2,4-F-Ph), 118.80, 118.77 (C5), 116.28 (d, $^2J_{C-C-O-P} = 3.8$ Hz, C2-Naph), 116.02 (d, $^2J_{C-C-O-P} = 2.5$ Hz, C2-Naph), 112.35, 112.32 (2d, $^2J_{C-F} = 21.0$ Hz, OCH₂-2,4-F-Ph), 104.75, 104.55 (2d, $^2J_{C-F} = 25.2$ Hz, OCH₂-2,4-F-Ph), 99.75, 99.72 (C8), 98.05 (C1'), 83.37, 83.21 (2d, $^3J_{C-C-O-P} = 7.5$ Hz, C4'), 80.18 (C2'), 76.04, 76.00 (C3'), 69.48, 69.34 (2d, $^2J_{C-O-P} = 5.0$ Hz, C5'), 61.18, 61.16 (OCH₂-2,4-F-Ph), 54.52, 54.42 (6OCH₃), 51.61, 51.58 (CHCH₃), 20.79, 20.70 (2'CCH₃), 20.40 (d, $^3J_{C-C-N-P} = 5.0$ Hz, CHCH₃), 20.36 (d, $^3J_{C-C-N-P} = 6.3$ Hz, CHCH₃).

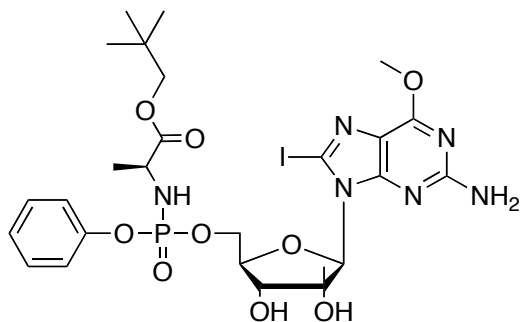
¹⁹F NMR (470 MHz, MeOD) δ -111.14, -113.69, 115.43, -117.41.

HPLC (System 1) $t_R = 19.29, 20.31$ min.

MS (ES+) m/z : 863.08 (MNa⁺, 100%).

HRMS C₃₂H₃₂N₆O₉F₂Na₁P₁I₁ Calculated: 863.0879 found: 863.0838.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(isopropoxy-L-methioninyl)] phosphate (9.6m).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and phenyl-(benzoxo-L-alaninyl) phosphorochloridate (**3.3n**, 463

mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using

CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6m** was obtained as an off white solid (54 mg, 11% yield).

³¹P NMR (202 MHz, MeOD) δ 3.79, 3.62.

¹H NMR (500 MHz, MeOD) δ 7.34-7.31 (m 1H, *Ph*), 7.27-7.21 (m, 2H, *Ph*), 7.18-7.10 (m, 2H, *Ph*), 5.98, 5.96 (2s, 1H, H_{1'}), 4.91-4.72 (m, 2H, H_{3'} and H_{5'a}), 4.60- 4.47 (m, 1H, H_{5'b}), 4.28- 4.20 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 4.00–3.94 (m, 1H, CHCH₃), 3.89, 3.86, 3.74, 3.68 (2AB, *J*_{AB} = 12.0 Hz, 2H, OCH₂C(CH₃)₃), 1.37, 1.23 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.06, 1.04 (2s, 3H, 2'CCH₃), 0.97, 0.92 (2s, 9H, OCH₂C(CH₃)₃).

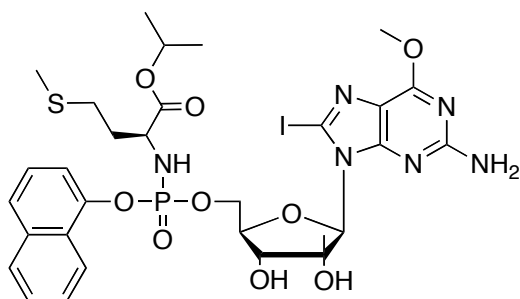
¹³C NMR (126 MHz, MeOD) δ 175.10 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 174.82 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.42, 161.25 (C6), 161.22 (C2), 154.58 (C4), 152.19, 152.14 (ipso *Ph*), 130.66, 130.58, 126.01, 125.94, 121.51, 121.47, 121.43 (CH-*Ph*), 119.13 (C5), 99.82 (C8), 98.07, 98.04 (C1'), 83.33, 83.10 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.22, 80.18 (C2'), 76.16, 76.01 (C3'), 75.27, 75.25 (OCH₂C(CH₃)₃), 69.29, 69.22 (2d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.26 (6OCH₃), 51.55, 51.47 (CHCH₃), 32.33, 32.27 (OCH₂C(CH₃)₃), 26.78 (OCH₂C(CH₃)₃), 20.72 (d, ³*J*_{C-C-N-P} = 5.0 Hz, CHCH₃), 20.67 (d, ³*J*_{C-C-N-P} = 7.5 Hz, CHCH₃), 20.36 (2'CCH₃).

HPLC (System 1) *t*_R = 18.27 min.

MS (ES+) *m/z*: 757.12 (M+Na⁺, 100%).

HRMS C₂₆H₃₇N₆O₉P₁I₁Na Calculated: 757.1224 found: 757.1248.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(isopropoxy-*L*-methioninyl)] phosphate (9.6m**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol), and α-naphthyl-(isopropoxy-*L*-

methioninyl) phosphorochloridate (**3.3x**, 570 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 3%, gradient), the pure compound **9.6m** was obtained as an off white solid (109 mg, 14% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.38, 3.92.

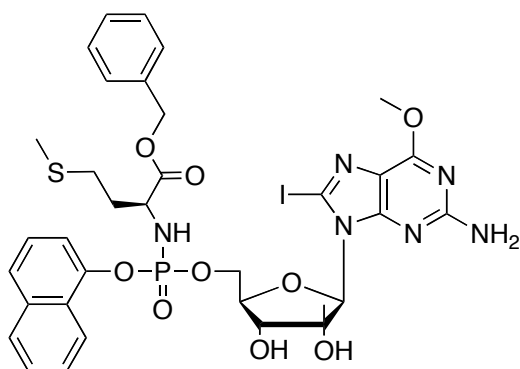
^1H NMR (500 MHz, MeOD) δ 8.20-8.18, 8.00-7.99 (2m, 1H, H₈-Naph), 7.87- 7.85, 7.78-7.76 (2m, 1H, H₅-Naph), 7.67, 7.56 (2d, J = 8.5 Hz, 1H, H₄-Naph), 7.53– 7.38 (m, 3H, H₂, H₇, H₆-Naph), 7.35-7.26 (m, 1H, H₃-Naph), 5.98, 5.97 (2s, 1H, H_{1'}), 4.91-4.78 (m, 4H, H_{3'} and H_{5'a} and OCH(CH₃)₂), 4.63-4.56 (m, 1H, H_{5'b}), 4.31, 4.25 (2td, J =8.5, 2.5 Hz, 1H, H_{4'}), 4.05-3.98 (m, 1H, CHCH₂CH₂SCH₃), 4.01, 3.97 (2s, 3H, 6OCH₃), 2.37-2.33 (m, 2H, CHCH₂CH₂SCH₃), 1.94, 1.92 (2s, 3H, CHCH₂CH₂SCH₃), 1.91-1.73 (m, 2H, CHCH₂CH₂SCH₃), 1.14-1.10 (m, 6H, OCH(CH₃)₂), 1.03, 1.02 (s, 3H, 2'CCH₃).

^{13}C NMR (126 MHz, MeOD) δ 173.83, 173.64 (C=O), 161.37, 161.26 (C6), 161.18, 161.04 (C2), 154.51, 154.44 (C4), 148.05 (d, $^2J_{\text{C-O-P}}$ = 7.5 Hz, ipso Naph), 147.92 (d, $^2J_{\text{C-O-P}}$ = 6.3 Hz, ipso Naph), 136.24, 136.08 (C10-Naph), 128.79, 128.64, (CH Naph), 127.86 (d, $^3J_{\text{C-C-O-P}}$ = 6.3 Hz, C9-Naph), 127.74 (d, $^3J_{\text{C-C-O-P}}$ = 5.0 Hz, C9-Naph), 127.57, 127.39, 127.16, 126.45, 126.32, 125.81, 125.62, 122.89, 122.75 (CH Naph), 119.16 (C5), 116.11, 116.88 (2d, $^3J_{\text{C-C-O-P}}$ = 2.5 Hz, C2-Naph), 99.80 (C8), 98.02 (C1'), 83.36, 83.16 (2d, $^3J_{\text{C-C-O-P}}$ = 7.5 Hz, C4'), 80.26, 80.20 (C2'), 76.14, 76.02 (C3'), 70.36, 70.34 (OCH(CH₃)₂), 69.66 (d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C5'), 69.62 (d, $^2J_{\text{C-O-P}}$ = 6.3 Hz, C5'), 55.12 (CHCH₂CH₂SCH₃), 54.26, 54.21 (6OCH₃), 34.56 (d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, CHCH₂CH₂SCH₃), 34.39 (d, $^3J_{\text{C-C-N-P}}$ = 7.5 Hz, CHCH₂CH₂SCH₃), 30.82, 30.79 (CHCH₂CH₂SCH₃), 22.02, 21.91 (OCH(CH₃)₂), 20.75, 30.68 (2'CCH₃), 15.26, 15.23 (CHCH₂CH₂SCH₃).

MS (ES+) m/z : 617.12 (MH⁺, 100%).

HRMS C₃₀H₃₉N₆O₉P₁S₁I₁ Calculated: 617.1257 found: 617.1248.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(benzoxy-L-methioninyl)] phosphate (9.6n).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.67 mmol), *t*BuMgCl (1.37 ml, 1.37 mmol),

and α -naphthyl-(benzoxy-L-methioninyl) phosphorochloridate (**3.3z**, 640 mg, 1.37 mmol) in 5 mL of THF. After silica gel column chromatography using $\text{CHCl}_3/\text{MeOH}$ (0 to 3%, gradient), the pure compound **9.6m** was obtained as an off white solid (180 mg, 14% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.17, 3.61.

^1H NMR (500 MHz, MeOD) δ 8.18-8.16, 8.00-7.99 (2m, 1H, $\text{H}_8\text{-Naph}$), 7.85- 7.83, 7.76-7.75 (2m, 1H, $\text{H}_5\text{-Naph}$), 7.64, 7.55 (2d, $J = 8.5$ Hz, 1H, $\text{H}_4\text{-Naph}$), 7.50- 7.41 (m, 3H, $\text{H}_2, \text{H}_7, \text{H}_6\text{-Naph}$), 7.36-7.29 (m, 1H, $\text{H}_3\text{-Naph}$), 7.26- 7.23 (m, 5H, OCH_2Ph), 5.99, 5.98 (2s, 1H, $\text{H}_{1'}$), 5.03- 4.87 (m, 2H, OCH_2Ph), 4.85- 4.80 (m, 2H, $\text{H}_{3'}$ and $\text{H}_{5'a}$), 4.65- 4.57 (m, 1H, $\text{H}_{5'b}$), 4.34- 4.24 (m, 1H, $\text{H}_{4'}$), 4.16- 4.68 (m, 1H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 3.98, 3.95 (2s, 3H, 6OCH_3), 2.28 (t, $J = 7.0$ Hz, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.89- 1.75 (m, 2H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 1.86, 1.83 (2s, 3H, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$) 1.03 (s, 3H, $2'\text{CCH}_3$).

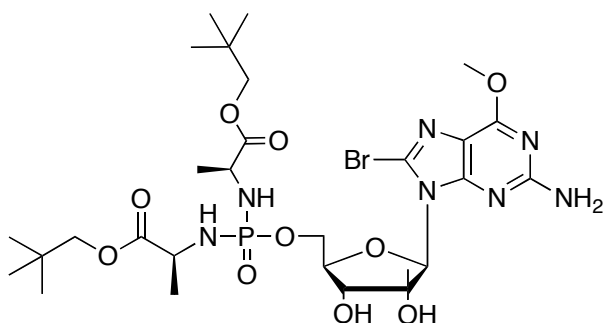
^{13}C NMR (126 MHz, MeOD) δ 174.04, 173.86 (2d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, C=O), 161.37, 161.26 (C6), 161.17, 161.03 (C2), 154.53, 154.45 (C4), 148.01 (d, $^2J_{\text{C-O-P}} = 7.5$ Hz, ipso Naph), 147.93 (d, $^2J_{\text{C-O-P}} = 6.3$ Hz, ipso Naph), 137.05, 137.01 (ipso OCH_2Ph), 136.22, 136.06 (C10-Naph), 129.67, 129.56, 129.54, 129.34, 129.32, 129.28, 128.81, 128.66 (CH-Naph and OCH_2Ph), 127.85 (d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.75 (d, $^3J_{\text{C-C-O-P}} = 5.0$ Hz, C9-Naph), 127.60, 127.45, 127.23, 126.48, 126.37, 125.86, 125.68, 122.89, 122.75 (CH-Naph), 119.19, 119.14 (C5), 116.19 (d, $^3J_{\text{C-C-O-P}} = 3.8$ Hz, C2-Naph), 115.98 (d, $^3J_{\text{C-C-O-P}} = 2.5$ Hz, C2-Naph), 99.79 (C8), 98.02 (C1'), 83.37, 83.15 (2d, $^3J_{\text{C-C-O-P}} = 7.5$ Hz, C4'), 80.27, 80.22 (C2'), 76.14, 76.05 (C3'), 69.64 (d, $^2J_{\text{C-O-P}} = 6.3$ Hz, C5'), 69.59 (d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 67.99 (OCH_2Ph), 55.02 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 54.29, 54.25 (6OCH_3), 34.44 (d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 34.33 (d, $^3J_{\text{C-C-N-P}} = 7.5$ Hz, $\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 30.68 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$), 20.79, 20.72 ($2'\text{CCH}_3$), 15.22 ($\text{CHCH}_2\text{CH}_2\text{SCH}_3$).

HPLC (system 1) $t_R = 19.93, 20.75$ min.

MS (ES+) m/z : 865.13 (MH^+ , 100%).

HRMS $\text{C}_{304}\text{H}_{39}\text{N}_6\text{O}_{10}\text{P}_1\text{S}_1\text{I}_1$ Calculated: 865.1282 found: 865.1256.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (9.7a).



8.2e (200 mg, 0.30 mmol) was dissolved in MeOH (10 ml) and NBS (53 mg, 0.30 mmol) was added. The mixture was stirred at room temperature for 18 h. After that time, solution was concentrated and the

resulting solid was purified by silica gel chromatography using 1-4% (gradient) of MeOH in CHCl₃ to yield the desired product **9.7a** as a white solid (56 mg, 25% yield).

³¹P NMR (202 MHz, MeOD) δ 13.87.

¹H NMR (500 MHz, MeOD) δ 6.04 (s, 1H, H_{1'}), 4.91-4.80 (m, 2H, H_{3'}), 4.63- 4.58 (m, 1H, H_{5'a}), 4.41- 4.37 (m, 1H, H_{5'b}), 4.22- 4.18 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.97-3.88 (m, 2H, 2x CHCH₃), 3.77, 3.76, 3.66, 3.60 (2AB, JAB= 10.5 Hz, 4H, 2x OCH₂C(CH₃)₃), 1.38 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.25 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.06 (s, 3H, 2'CCH₃), 0.96 (s, 9H, OCH₂C(CH₃)₃), 0.91 (s, 9H, OCH₂C(CH₃)₃).

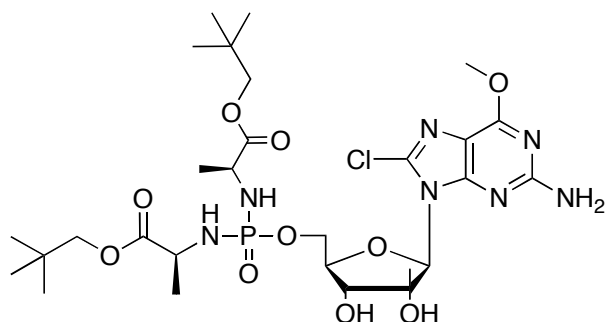
¹³C NMR (126 MHz, MeOD) δ 175.72 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 175.53 (d, ³*J*_{C-C-N-P} = 7.5 Hz, C=O), 161.63 (C6), 161.47 (C2), 154.94 (C4), 125.83 (C8), 116.23 (C5), 95.86 (C1'), 83.40 (d, ³*J*_{C-C-O-P} = 6.3 Hz, C4'), 80.24 (C2'), 76.00 (C3'), 75.34, 75.29 (OCH₂C(CH₃)₃), 67.61 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.30 (6OCH₃), 51.05 (CHCH₃), 50.91 (CHCH₃), 32.34 (OCH₂C(CH₃)₃), 32.27 (OCH₂C(CH₃)₃), 26.84 (OCH₂C(CH₃)₃), 26.78 (OCH₂C(CH₃)₃), 21.07 (d, ³*J*_{C-C-N-P} = 5.0 Hz, CHCH₃), 21.01 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.60 (2'CCH₃).

HPLC (System 1) *t*_R = 20.67 min.

MS (ES+) *m/z*: 754.24 (M+NH⁺, 100%).

HRMS C₂₈H₄₈N₇O₁₀P₁Br₁ calculated: 752.2384 found 752.2379.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (9.8a).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-8-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.2**, 300 mg, 0.87 mmol), POCl₃ (0.28 ml, 0.87 mmol), Et₃N (0.12 ml, 0.87

mmol), L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 1.44 g, 4.36 mmol), Et₃N (1.20 ml, 8.72 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **9.8a** as a white foam (160 mg, 26%).

³¹P NMR (202 MHz, MeOD) δ 13.87.

¹H NMR (500 MHz, MeOD) δ 6.03 (s, 1H, H_{1'}), 4.78- 4.76 (m, 2H, H_{3'}), 4.62- 4.57 (m, 1H, H_{5'a}), 4.43- 4.38 (m, 1H, H_{5'b}), 4.23- 4.19 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.99-3.93 (m, 1H, CHCH₃), 3.92-3.87 (m, 1H, CHCH₃), 3.79, 3.78, 3.68, 3.61 (2AB, J_{AB} = 10.5 Hz, 4H, 2x OCH₂C(CH₃)₃), 1.38 (d, J = 7.0 Hz, 3H, CHCH₃), 1.25 (d, J = 7.0 Hz, 3H, CHCH₃), 1.07 (s, 3H, 2'CCH₃), 0.95 (s, 9H, OCH₂C(CH₃)₃), 0.90 (s, 9H, OCH₂C(CH₃)₃).

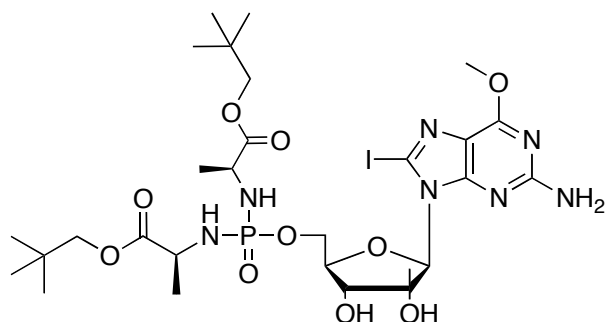
¹³C NMR (126 MHz, MeOD) δ 175.71 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, C=O), 175.53 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, C=O), 161.78 (C6), 161.48 (C2), 154.66 (C4), 136.71 (C8), 114.40 (C5), 94.63 (C1'), 83.40 (d, $^3J_{C-C-O-P}$ = 6.3 Hz, C4'), 80.22 (C2'), 75.96 (C3'), 75.36, 75.31 (CH₂ ester), 67.55 (d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 54.42 (6OCH₃), 51.06 (CHCH₃), 50.91 (CHCH₃), 32.36 (OCH₂C(CH₃)₃), 32.29 (OCH₂C(CH₃)₃), 26.88 (OCH₂C(CH₃)₃), 26.82 (OCH₂C(CH₃)₃), 21.15 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 21.08 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, OCH₂C(CH₃)₃), 20.61 (2'CCH₃).

HPLC (System 1) t_R = 20.67 min.

MS (ES+) m/z : 708.29 (M⁺, 100%).

HRMS C₂₈H₄₈N₇O₁₀P₁Cl₁ calculated: 708.2889 found 708.2872.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(2,2-dimethylpropoxy-L-alaninyl) phosphate (9.9a).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.69 mmol), POCl₃ (0.06 ml, 0.69 mmol), Et₃N (0.09 ml, 0.69

mmol), L-alanine 2,2-dimethylpropyl ester tosylate salt (**3.2g**, 1.14 g, 3.43 mmol), Et₃N (0.96 ml, 6.86 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **9.9a** as a white foam (50 mg, 10%).

³¹P NMR (202 MHz, MeOD) 13.89.

¹H NMR (500 MHz, MeOD) 5.98 (s, 1H, H_{1'}), 4.91- 4.83 (m, 2H, H_{3'}), 4.63- 4.57 (m, 1H, H_{5'a}), 4.42- 4.38 (m, 1H, H_{5'b}), 4.21- 4.18 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.97-3.90 (m, 2H, CHCH₃), 3.78, 3.75, 3.67, 3.61 (2AB, *J*_{AB}= 10.5 Hz, 4H, 2x OCH₂C(CH₃)₃), 1.38 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.24 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.04 (s, 3H, 2'CCH₃), 0.96 (s, 9H, OCH₂C(CH₃)₃), 0.91 (s, 9H, OCH₂C(CH₃)₃).

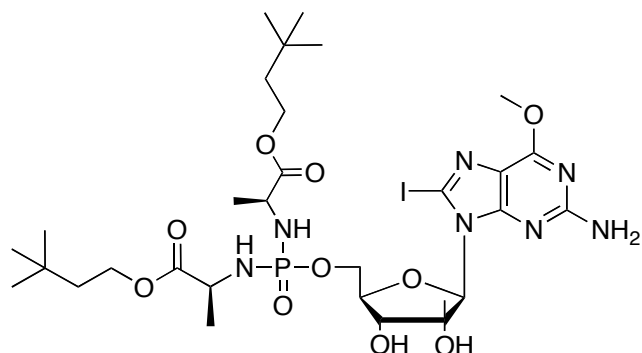
¹³C NMR (126 MHz, MeOD) δ 175.74 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 175.55 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.40 (C6), 161.27 (C2), 154.64 (C4), 119.12 (C5), 99.76 (C8), 98.00 (C1'), 83.35 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.29 (C2'), 76.07 (C3'), 75.35, 75.29 (2x OCH₂C(CH₃)₃), 67.66 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.24 (6OCH₃), 51.05 (CHCH₃), 50.92 (CHCH₃), 32.36 (OCH₂C(CH₃)₃), 32.29 (OCH₂C(CH₃)₃), 26.87 (OCH₂C(CH₃)₃), 26.80 (OCH₂C(CH₃)₃), 21.07 (CHCH₃), 21.03 (CHCH₃), 20.78 (2'CCH₃).

HPLC (System 1) *t*_R = 15.12 min.

MS (ES⁺) *m/z*: 800.22 (M+Na⁺, 100%).

HRMS C₂₈H₄₈N₇O₁₀P₁I₁ calculated: 800.2245 found 800.2212.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(3,3-dimethyl-1-butoxy-L-alaninyl) phosphate (9.9b**).**



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.69 mmol), POCl₃ (0.06 ml, 0.69 mmol),

Et₃N (0.09 ml, 0.69 mmol), L-alanine 3,3-dimethyl-1-butyl ester tosylate salt (**3.2i**, 1.14 g, 3.43 mmol), Et₃N (0.96 ml, 6.86 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **9.9b** as a white foam (130 mg, 23%).

³¹P NMR (202 MHz, MeOD) 13.96.

¹H NMR (500 MHz, MeOD) 5.98 (s, 1H, H_{1'}), 4.89- 4.81 (m, 2H, H_{3'}), 4.63- 4.57 (m, 1H, H_{5'a}), 4.41-4.37 (m, 1H, H_{5'b}), 4.21-4.09 (m, 5H, H_{4'} and 2x OCH₂CH₂C(CH₃)₃), 4.04 (s, 3H, 6OCH₃), 3.92-3.86 (m, 1H, CHCH₃), 3.85-3.80 (m, 1H, CHCH₃), 1.57 (t, *J*= 7.0 Hz, 2H, OCH₂CH₂C(CH₃)₃), 1.52 (t, *J*= 7.0 Hz, 2H, OCH₂CH₂C(CH₃)₃), 1.34 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.20 (d, *J*= 7.0 Hz, 3H, CHCH₃), 1.03 (s, 3H, 2'CCH₃), 0.97 (s, 9H, OCH₂CH₂C(CH₃)₃), 0.94 (s, 9H, OCH₂CH₂C(CH₃)₃).

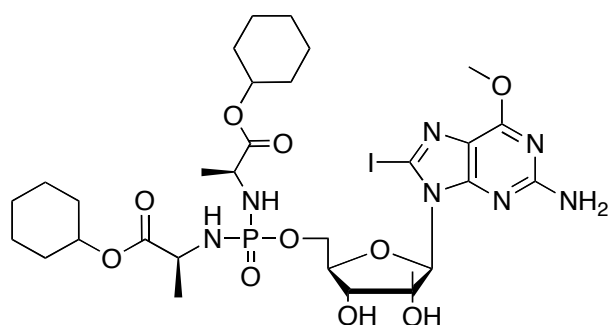
¹³C NMR (126 MHz, MeOD) δ 175.74 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 175.56 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 161.44 (C6), 161.17 (C2), 154.58 (C4), 119.13 (C5), 99.72 (C8), 97.98 (C1'), 83.33 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.34 (C2'), 76.13 (C3'), 67.57 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 63.95 (OCH₂CH₂C(CH₃)₃), 63.89 (OCH₂CH₂C(CH₃)₃), 54.23 (6OCH₃), 51.03 (CHCH₃), 50.91 (CHCH₃), 42.87 (OCH₂CH₂C(CH₃)₃), 30.63 (OCH₂CH₂C(CH₃)₃), 30.61 (OCH₂CH₂C(CH₃)₃), 30.13 (OCH₂CH₂C(CH₃)₃), 30.11 (OCH₂CH₂C(CH₃)₃), 20.90 (CHCH₃), 20.85 (CHCH₃), 20.62 (2'CCH₃).

HPLC (System 1) *t*_R = 22.51 min.

MS (ES+) *m/z*: 828.25 (M+Na⁺, 100%).

HRMS C₃₀H₅₂N₇O₁₀P₁I₁ calculated: 828.2558 found 828.2524.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(cyclohexoxy-L-alaninyl) phosphate (9.9c**).**



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.69 mmol), POCl₃ (0.06 ml, 0.69 mmol), Et₃N (0.09 ml, 0.69

mmol), L-alanine cyclohexyl ester hydrochloride salt (**3.2c**, 0.72 g, 3.43 mmol), Et₃N (0.96 ml, 6.86 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **9.9c** as a white foam (191 mg, 34%).

³¹P NMR (202 MHz, MeOD) 13.97.

¹H NMR (500 MHz, MeOD) 5.98 (s, 1H, H_{1'}), 4.91-82 (m, 2H, H_{3'}), 4.72-4.67 (m, 1H, OCH ester), 4.63- 4.57 (m, 2H, H_{5'a} and OCH ester), 4.42- 4.38 (m, 1H, H_{5'b}), 4.22- 4.18 (m, 1H, H_{4'}), 4.04 (s, 3H, 6OCH₃), 3.87 (q, *J* = 7.0 Hz, 1H, CHCH₃), 3.82 (q, *J* = 7.0 Hz, 1H, CHCH₃), 1.84-1.69 (m, 8H, 4xCH₂ ester), 1.41-1.29 (m, 12H, 6xCH₂ ester), 1.34 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.21 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.04 (s, 3H, 2'CCH₃).

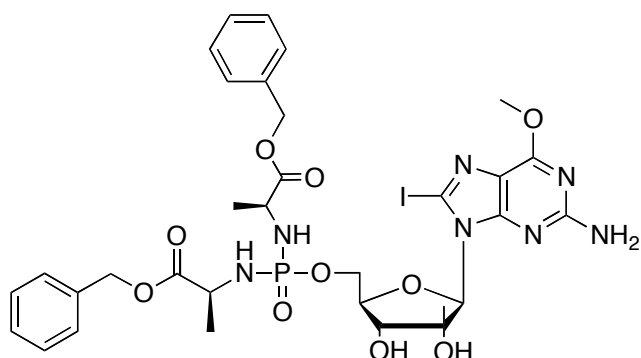
¹³C NMR (126 MHz, MeOD) δ 175.23 (d, ³*J*_{C-C-N-P} = 6.3Hz, C=O), 174.96 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.40 (C6), 161.20 (C2), 154.58 (C4), 119.09 (C5), 99.82 (C8), 97.98 (C1'), 83.39 (d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.31 (C2'), 76.05 (C3'), 74.80 (OCH ester), 74.69 (OCH ester), 67.62 (d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.34 (6OCH₃), 51.13 (CHCH₃), 50.94 (CHCH₃), 26.47 (CH₂ ester), 26.44 (CH₂ ester), 24.71 (CH₂ ester), 24.68 (CH₂ ester), 21.07 (CHCH₃), 21.03 (CHCH₃), 20.78 (2'CCH₃)

HPLC (System 1) *t*_R = 20.91 min

MS (ES+) *m/z*: 824.23 (M+H⁺, 100%).

HRMS C₃₀H₄₈N₇O₁₀P₁I₁ calculated: 824.2245 found 824.2277.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-bis(benzyl-L-alaninyl) phosphate (9.9d).



Prepared according to the Standard Procedure 13a, from: 2-amino-6-*O*-methyl-8-*C*-iodo-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.3**, 300 mg, 0.69 mmol), POCl₃ (0.06 ml, 0.69 mmol), Et₃N (0.09 ml, 0.69 mmol), L-

alanine benzyl ester tosylate salt (1.21 g, 3.43 mmol), Et₃N (0.96 ml, 6.86 mmol) in 10 ml of 1:1 mixture of dry THF and DCM. The crude mixture was purified by column chromatography in gradient (CHCl₃/MeOH 0 to 5%), to give a pure product **9.9d** as a white foam (230 mg, 40%).

³¹P NMR (202 MHz, MeOD) δ 13.86.

¹H NMR (500 MHz, MeOD) δ 7.35-7.23 (m, 10H, 2Ph), 6.01 (2s, 1H, H_{1'}), 5.09, 5.05, 4.94, 4.88 (2AB, J_{AB} = 12.0 Hz, 2H, CH₂ ester), 4.88- 4.85 (m, 1H, H_{3'}), 4.59-4.56 (m, 1H, H_{5'a}), 4.47- 4.43 (m, 1H, H_{5'b}), 4.25- 4.21 (m, 1H, H_{4'}), 4.01 – 3.88 (m, 2H, 2xH α , Ala), 3.99 (s, 3H, 6OCH₃), 1.31 (d, J = 7.0 Hz, 3H, CH₃ Ala), 1.20 (d, J = 7.5 Hz, 3H, CH₃ Ala), 1.04 (s, 3H, 2'CCH₃).

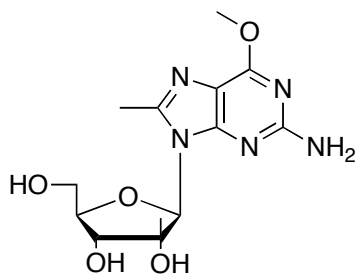
¹³C NMR (126 MHz, MeOD) δ 175.55 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O ester), 175.44 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O ester), 161.44 (C6), 161.15 (C2), 154.52 (C4), 137.28, 137.19 (ipso Ph), 129.99, 129.67, 129.64, 129.44, 129.36, 129.27, 128.35, 128.07 (C-Ph), 119.17 (C5), 99.89 (C8), 97.98 (C1'), 83.35 (d, $^3J_{C-C-O-P}$ = 6.3 Hz, C4'), 80.39 (C2'), 76.16 (C3'), 67.93 (d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 67.60, 67.57 (CH₂ ester), 54.49 (6OCH₃), 51.09, 51.01 (2C α Ala), 21.28 (2'CCH₃), 20.93 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, CH₃ Ala), 20.84 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, CH₃ Ala)

HPLC t_R = 18.60 min

MS (ES⁺) m/z : 862.22 (M+Na⁺, 100%).

HRMS C₃₂H₄₀N₇O₁₀P₁I₁ calculated: 840.1619 found 840.1636.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (9.13).



9.1 (1.00 g, 2.56 mmol) in an excess of HMDS (40 ml) in the presence of 1,4-dioxane (15 ml) and a catalytic amount of $(\text{NH}_4)_2\text{SO}_4$ was refluxed for 3-4 h (TLC shows completion of reaction) under argon atmosphere. After that time solvents were removed under reduced pressure. The reaction mixture was dissolved in dry THF (20 ml) and Tetrakis (0.44 g, 0.38 mmol) was added followed by the addition of AlMe_3 (2.67 ml, 5.16 mmol). The reaction mixture was stirred at reflux for 18 h. After that time, solvent was removed under vacuum and the residue was dissolved in MeOH and NH_4Cl (0.80 g, 14.95 mmol) was added. The reaction mixture was stirred at reflux for 2 h. After that time solvent was evaporated under reduced pressure and the crude mixture was purified on silica gel, using $\text{CHCl}_3/\text{MeOH}$ (0 to 5%, gradient) as an eluent, to give pure **9.13** (0.60 g, 72%) as a white solid.

^1H NMR (500 MHz, DMSO) δ 6.69 (bs, 2H, NH_2), 5.75 (s, 1H, $\text{H}_{1'}$), 5.12 (s, 1H, $2'\text{OH}$), 5.08 (t, $J = 5.0$ Hz, 1H, $5'\text{OH}$), 4.99 (d, $J = 7.0$ Hz, 1H, $3'\text{OH}$), 4.47-4.29 (m, 1H, $\text{H}_{3'}$), 3.93 (s, 3H, 6OCH_3), 3.89-3.87 (m, 1H, $\text{H}_{4'}$), 3.77- 3.68 (m, 2H, $\text{H}_{5'}$), 2.43 (s, 3H, 8CCH_3), 0.83 (s, 3H, $2'\text{CCH}_3$).

^{13}C NMR (126 MHz, DMSO) δ 162.93 (C-6), 161.35 (C-2), 155.00 (C-4), 151.59 (C-8), 131.49, 131.06 (CH Ph), 130.53 (ipso Ph), 130.05 (CH Ph), 115.53 (C-5), 95.27 (C-1'), 84.16 (C-4'), 80.30 (C-2'), 74.78 (C-3'), 62.74 (C-5'), 54.30 (6OCH_3), 21.39 ($2'\text{CCH}_3$).

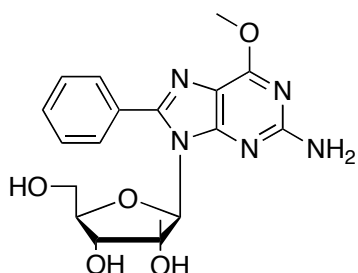
HPLC (System 1) $t_R = 5.68$ min.

HPLC (System 2) $t_R = 10.96$ min.

MS (ES+) m/z : 326.15 ($\text{M} + \text{H}^+$, 100%);

HRMS $\text{C}_{13}\text{H}_{20}\text{N}_5\text{O}_5$ calculated: 326.1464 found: 326.1476.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-phenyl-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (9.14).



Palladium acetate (4.2 mg, 2.5mol%), TPPTS (30.5 mg, 6.5mol%), sodium carbonate (164 mg, 1.54 mol), **9.1** (300 mg, 0.77 mmol) and phenylboronic acid (140.5 mg, 1.15 mmol) were placed in a round bottom flask under nitrogen. Degassed mixture of H₂O:ACN (2:1) was added and the reaction mixture was heated at 80 °C for 4 h. After that time, the reaction mixture was diluted with water and pH was adjusted to 6-7 with 10% aqueous HCl. The reaction mixture was then heated to dissolve precipitated solids and then allowed to cool down to 0 °C and stand at 0 °C for 16 h. After that time pure product was filtered off and dried in air. The pure product was obtained as a white solid (220 mg, 74%).

¹H NMR (500 MHz, MeOD) 7.68-7.66 (m, 2H, *Ph*), 7.60-7.58 (m, 3H, *Ph*), 5.96 (s, 1H, H_{1'}), 4.80 (d, *J*=8.5 Hz, 1H, H_{3'}), 4.09 (s, 3H, 6OCH₃), 4.02-3.94 (m, 3H, H_{4'} and H_{5'}), 1.15(s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 159.90 (C6), 158.79 (C2), 154.04 (C4), 146.73 (C8), 112.84 (C5), 92.57 (C1'), 82.95 (C4'), 79.10 (C2'), 73.51 (C3'), 61.13 (C5'), 53.10 (6OCH₃), 20.61 (2'CCH₃), 15.01 (8CCH₃).

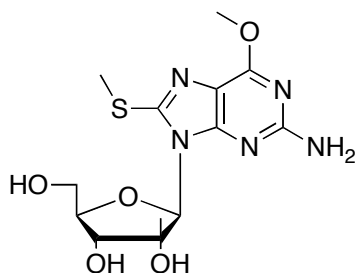
HPLC (System 1) *t*_R= 9.92 min.

HPLC (System 2) *t*_R=16.80 min.

MS (ES⁺) *m/z*: 388.16 (M+H⁺, 100%).

HRMS C₁₈H₂₂N₅O₅ calculated: 388.1621 found: 388.1628.

Synthesis of 2-Amino-6-*O*-methyl-8-*C*-thiomethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (9.15).



To a solution of 2-amino-6-*O*-methyl-8-*C*-bromo-9-(2'-*C*-methyl- β -D-ribofuranosyl)purine (**9.1**, 0.40 g, 1.03 mmol) in dry DMF (30 ml) was aqueous solution of sodium methanethiolate (0.40 g in 2 ml of H₂O, 6.18

mmol. The reaction mixture was stirred at ambient temperature for 2 h, after that time mixture was diluted with water (50 ml) and extracted 3x with EtOAc (3x 20 ml). Combined organic fractions were dried over anhydrous MgSO_4 and evaporated to dryness under reduced pressure. The residue was purified by silica gel chromatography ($\text{CHCl}_3/\text{MeOH}$, 95:5) to give the pure compound **9.15** (0.38 g, 98%) as a white solid.

^1H NMR (500 MHz, MeOD) 5.97 (s, 1H, $\text{H}_{1'}$), 4.54 (d, $J=8.5$ Hz, 1H, $\text{H}_{3'}$), 4.07-3.97 (m, 3H, $\text{H}_{4'}$ and $\text{H}_{5'}$), 4.04 (s, 3H, 6OCH_3), 2.71 (s, 3H, 8CSCH_3), 0.99 (s, 3H, $2'\text{CCH}_3$).

^{13}C NMR (126 MHz, MeOD) δ 161.27 (C6), 160.52 (C2), 155.51 (C4), 149.86 (C8), 116.37 (C5), 95.13 (C1'), 84.21 (C4'), 80.34 (C2'), 74.746 (C3'), 62.31 (C5'), 54.38 (6OCH_3), 20.98 ($2'\text{CCH}_3$), 9.28 (8CSCH_3).

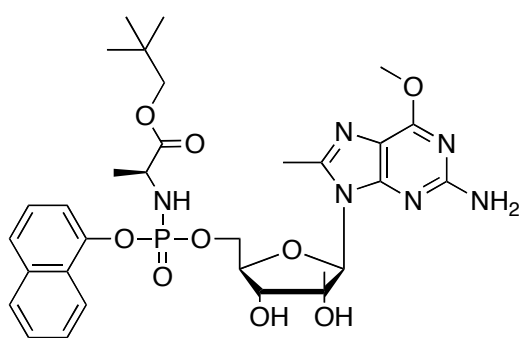
HPLC (System 1) t_R = 8.00 min.

HPLC (System 2) t_R = 14.75 min.

MS (ES+) m/z : 358.12 ($\text{M}+\text{H}^+$, 100%).

HRMS $\text{C}_{13}\text{H}_{20}\text{N}_5\text{O}_5\text{S}$ calculated: 358.1185 found: 358.1186.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (9.17a**).**



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.13**, 200 mg, 0.54 mmol), $t\text{BuMgCl}$ (1.07 ml, 1.07 mmol), and α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 356

mg, 1.07 mmol) in 5 mL of THF. After silica gel column chromatography using $\text{CHCl}_3/\text{MeOH}$ (0 to 7%, gradient), the pure compound **9.17a** was obtained as an off white solid (50 mg, 14% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.18, 3.77.

^1H NMR (500 MHz, MeOD) δ 8.17-8.15, 8.04-8.02 (2m, 1H, H₈-Naph), 7.87-7.85, 7.80-7.78 (2m, 1H, H₅-Naph), 7.67, 7.58 (2d, J = 8.0 Hz, 1H, H₄-Naph), 7.52-7.44 (m, 3H, H₂, H₇, H₆-Naph), 7.34-7.28 (m, 1H, H₃-Naph), 5.84 (2s, 1H, H_{1'}), 4.82-4.77 (m, 2H, H_{3'} and H_{5'a}), 4.65-4.54 (m, 1H, H_{5'b}), 4.30-4.23 (m, 1H, H_{4'}), 4.08-4.03 (m, 1H, CHCH₃), 4.00, 3.99 (2s, 3H, 6OCH₃), 3.74, 3.71, 3.63, 3.57 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 2.53 (s, 3H, 8CCH₃), 1.32, 1.25 (2d, J = 7.0 Hz, 3H, CHCH₃), 1.01 (2s, 3H, 2'CCH₃), 0.86, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

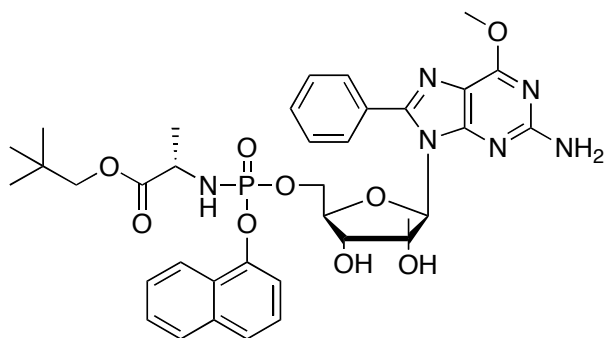
^{13}C NMR (126 MHz, MeOD) δ 175.04 (d, $^3J_{C-C-N-P}$ = 5.0 Hz, C=O), 174.74 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, C=O), 161.77, 161.73 (C6), 161.07, 160.99 (C2), 155.07 (C4), 149.14, 149.10 (C8), 148.02, 147.96 (ipso Naph), 136.25, 136.15 (C10-Naph), 128.80, 128.67 (CH-Naph), 127.86, 127.82 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.68, 127.55, 127.36, 127.18, 126.47, 127.37, 125.82, 125.65 (CH-Naph), 122.81, 122.73 (C5), 116.25, 115.94 (2d, $^3J_{C-C-O-P}$ = 3.3 Hz, C2-Naph), 114.30 (CH-Naph), 94.23, 94.19 (C1'), 83.06, 82.85 (2d, $^3J_{C-C-O-P}$ = 7.5 Hz, C4'), 80.07 (C2'), 76.02, 75.97 (C3'), 75.33, 75.28 (OCH₂C(CH₃)₃), 69.37, 69.33 (C5'), 54.01 (6OCH₃), 51.66, 51.62 (CHCH₃), 32.22, 32.27 (OCH₂C(CH₃)₃), 26.70, 26.67 (OCH₂C(CH₃)₃), 20.81 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.69 (d, $^3J_{C-C-N-P}$ = 7.5 Hz, CHCH₃), 20.63, 20.54 (2'CCH₃), 14.77, 14.69 (8CCH₃).

HPLC (System 1) t_R = 18.60, 19.17 min.

MS (ES+) m/z : 673.28 (M+H⁺, 100%).

HRMS C₃₁H₄₂N₆O₉P₁ Calculated: 673.2751 found: 673.2782.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-phenyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (9.18a).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-phenyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.14**, 250 mg, 0.65 mmol), *t*BuMgCl (1.29 ml, 1.29 mmol), and α -naphthyl-(2,2-

dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 427 mg, 1.29 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 7%,

gradient), the pure compound **9.18a** was obtained as an off white solid (35 mg, 7% yield).

^{31}P NMR (202 MHz, MeOD) δ 4.11, 3.64.

^1H NMR (500 MHz, MeOD) δ 8.15, 8.02 (2d, $J=8.0$ Hz, 1H, H₈-Naph), 7.84, 7.79 (2d, $J=8.0$ Hz, 1H, H₅-Naph), 7.67-7.64 (m, 3H, CH Ph and H₄-Naph), 7.60-7.58 (m, 3H, CH Ph), 7.51-7.43 (m, 3H, H₇, H₆, H₂-Naph), 7.38-7.27 (m, 1H, H₃-Naph), 5.93, 5.91 (2s, 1H, H_{1'}), 5.03 (d, $J=8.0$ Hz, 1H, H_{3'}), 4.80-4.72, 4.64-4.53 (2m, 2H, H_{5'}), 4.23, 4.17 (2td, $J=8.5, 2.5$ Hz, 1H, H_{4'}), 4.06-3.98 (m, 1H, CHCH₃), 3.92, 3.89 (2s, 3H, 6OCH₃), 3.73, 3.69, 3.61, 3.58 (2AB, $J_{\text{AB}}=10.5$ Hz, 2H, OCH₂C(CH₃)₃), 1.28, 1.24 (2d, $J=7.0$ Hz, 3H, CHCH₃), 1.18, 1.17 (2s, 3H, 2'CCH₃), 0.85, 0.83 (2s, 9H, OCH₂C(CH₃)₃).

^{13}C NMR (126 MHz, MeOD) δ 175.02 ($^3J_{\text{C-C-N-P}}=5.0$ Hz, C=O), 174.75 ($^3J_{\text{C-C-N-P}}=6.3$ Hz, C=O), 162.54 (C6), 161.35, 161.28 (C2), 155.14 (C4), 151.67, 151.63 (C8), 148.00, 147.95 (ipso Naph), 136.22, 136.14 (C10-Naph), 131.43, 131.12, 131.10 (CH Ph), 130.67, 130.05 (ipso Ph), 128.76, 128.68 (CH-Naph), 127.84, 127.79 (C9-Naph), 127.65, 127.57, 127.34, 127.23, 126.43, 126.37, 125.80, 125.66, 122.81, 122.70 (CH Naph), 116.24 (d, $^3J_{\text{C-C-O-P}}=3.8$ Hz, C2-Naph), 115.97 (d, $^3J_{\text{C-C-O-P}}=2.5$ Hz, C2-Naph), 115.52, 115.46 (C5), 94.76, 94.70 (C1'), 83.10 (d, $^3J_{\text{C-C-O-P}}=6.3$ Hz, 4'), 82.95 (d, $^3J_{\text{C-C-O-P}}=7.5$ Hz, C4'), 80.21, 80.19 (C2'), 76.14, 76.01 (C3'), 75.32 (OCH₂C(CH₃)₃), 69.74 (d, $^3J_{\text{C-C-O-P}}=6.3$ Hz, C5'), 69.63 (d, $^3J_{\text{C-C-O-P}}=5.0$ Hz, C5'), 54.21, 54.13 (6OCH₃), 51.64, 51.58 (CHCH₃), 32.24 (OCH₂C(CH₃)₃), 26.71, 26.66 (OCH₂C(CH₃)₃), 20.86 (d, $^3J_{\text{C-C-N-P}}=6.3$ Hz, CHCH₃), 20.68 (d, $^3J_{\text{C-C-N-P}}=7.5$ Hz, CHCH₃), 20.27, 20.23 (2'CCH₃).

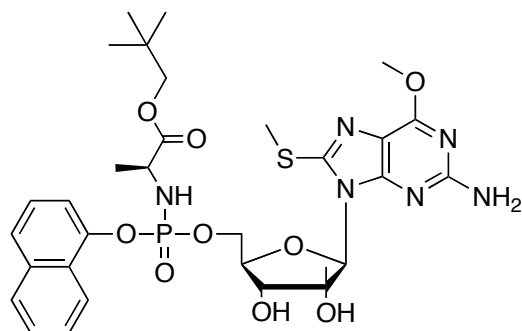
HPLC (System 1) $t_{\text{R}}=21.37, 22.12$ min.

HPLC (System 2) $t_{\text{R}}=27.55, 28.55$ min.

MS (TOF ES+) m/z : 735.29 ($\text{M}+\text{H}^+$, 100%).

HRMS C₃₆H₄₄N₆O₉P₁ calculated: 735.2907 found: 735.2900.

Synthesis of 2-amino-6-*O*-methyl-8-*C*-thiomethyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphate (9.18a).



Prepared according to the Standard Procedure 7: from 2-amino-6-*O*-methyl-8-*C*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**9.15**, 300 mg, 0.84 mmol), *t*BuMgCl (1.67 ml, 1.67mmol), and α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 556 mg, 1.67 mmol) in 5 mL of THF. After silica gel column chromatography using CHCl₃/MeOH (0 to 7%, gradient), the pure compound **9.17a** was obtained as an off white solid (139 mg, 23% yield).

³¹P NMR (202 MHz, MeOD) δ 4.18, 3.68.

¹H NMR (500 MHz, MeOD) δ 8.19-8.17, 8.06-8.04 (2m, 1H, H₈-Naph), 7.87- 7.82, 7.77-7.76 (2m, 1H, H₅-Naph), 7.67, 7.56 (2d, *J* = 8.0 Hz, 1H, H₄-Naph), 7.53-7.42(m, 3H, H₂, H₇, H₆-Naph), 7.38-7.27 (m, 1H, H₃-Naph), 5.99, 5.96 (2s, 1H, H_{1'}), 4.89-4.64 (m, 2H, H_{3'} and H_{5'a}), 4.60- 4.56 (m, 1H, H_{5'b}), 4.32-4.25 (m, 1H, H_{4'}), 4.10-4.03 (m, 1H, CHCH₃), 4.01, 3.99 (2s, 3H, 6OCH₃), 3.73, 3.70, 3.62, 3.56 (2AB, *J*_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 2.68, 2.66 (2s, 3H, 8CSCH₃), 1.32, 1.26 (2d, *J* = 7.0 Hz, 3H, CHCH₃), 1.03 (2s, 3H, 2'CCH₃), 0.84, 0.82 (2s, 9H, OCH₂C(CH₃)₃).

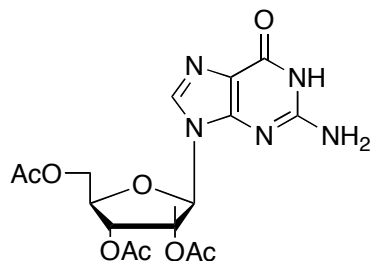
¹³C NMR (126 MHz, MeOD) δ 175.05 (d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 174.79 (d, ³*J*_{C-C-N-P} = 6.3 Hz, C=O), 161.00, 160.93 (C6), 161.78, 160.68 (C2), 155.99 (C4), 149.71 (C8), 148.04, 147.96 (ipso Naph), 136.23, 136.13 (C10-Naph), 128.83, 128.70, 127.93 (CH-Naph), 127.86, 127.80 (2d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-Naph), 127.78, 127.72, 127.60, 127.43, 127.23, 126.53, 126.43, 125.87, 125.68 (CH-Naph), 122.86, 122.76 (C5), 116.29, 115.96 (2d, ³*J*_{C-C-O-P} = 3.3 Hz, C2-Naph), 94.35 (C1'), 83.11, 82.89 (2d, ³*J*_{C-C-O-P} = 7.5 Hz, C4'), 80.14 (C2'), 76.15, 76.07 (C3'), 75.36, 75.31 (OCH₂C(CH₃)₃), 69.35, 69.31 (C5'), 54.25 (6OCH₃), 51.68, 51.63 (CHCH₃), 32.27, 32.24 (OCH₂C(CH₃)₃), 26.70, 26.67 (OCH₂C(CH₃)₃), 20.92 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.82 (d, ³*J*_{C-C-N-P} = 6.3 Hz, CHCH₃), 20.66, 20.59 (2'CCH₃), 15.57, 15.53 (8CSCH₃).

HPLC (System 1) t_R = 20.13, 21.05 min.

MS (ES+) m/z : 727.23 ($M+Na^+$, 100%).

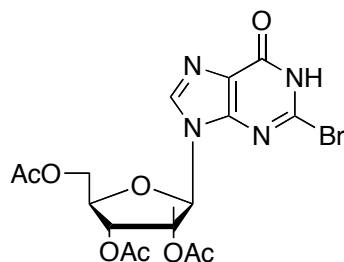
HRMS $C_{31}H_{42}N_6O_9P_1S_1$ Calculated: 705.2472 found: 705.2451.

12.9 Experimental section – Chapter Ten.

Synthesis of 2'-C-methylguanosine 2',3',5'-tri-*O*-acetate (**10.1**).

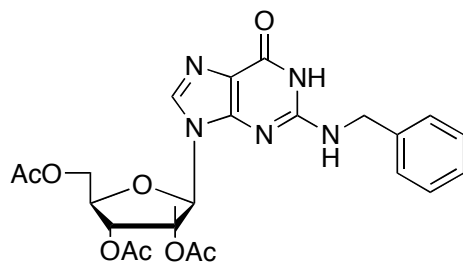
2'-C-methylguanosine (**4.10**, 2.00 g, 6.73 mmol) was suspended in dry acetonitrile (100 ml) and Et₃N (3.65 ml, 26.24 mmol) and DMAP (0.08 g, 0.67 mmol) were added under argon, followed by the dropwise addition of acetic anhydride (2.29 ml, 24.22 mmol). The reaction mixture was stirred at ambient temperature for 4 h. After that time reaction was concentrated to 1/3 volume and solids were filtered off washed with diethyl ether and dried. The pure compound **10.1** was obtained as a white solid (2.57 g, 90%).

¹H NMR (500 MHz, DMSO) δ 10.74 (bs, 1H, NH), 7.85 (s, 1H, H₈), 6.56 (bs, 2H, NH₂), 6.16 (s, 1H, H_{1'}), 5.45 (d, *J*=5.5 Hz, 1H, H_{3'}), 4.44-4.27 (m, 3H, H_{4'} and H_{5'}), 2.09 (s, 3H, CH₃ acetyl), 2.07 (s, 3H, CH₃ acetyl), 2.06 (s, 3H, CH₃ acetyl), 1.42 (s, 3H, 2'CCH₃).

Synthesis of 2-bromo-2'-C-methylxanthosine 2',3',5'-tri-*O*-acetate (**10.2**).

2'-C-methylguanosine 2',3',5'-tri-*O*-acetate (**10.1**, 1.50 g, 3.54 mmol), isopenyl nitrite (9.48 ml, 70.86 mmol) and bromoform (63 ml) were heated at 95 °C for 3 h. After that time volatiles were evaporated under reduced pressure and the residue was purified on silica gel using EtOAc/MeOH (0 to 5%, gradient) as an eluent, to give pure product **10.2** as yellowish solid (0.60 g, 35%).

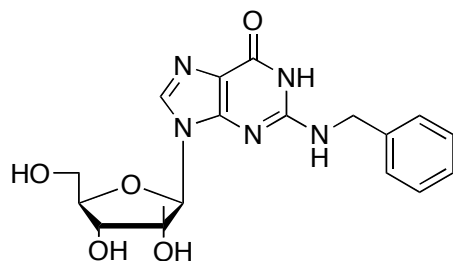
¹H NMR (500 MHz, DMSO) δ 13.42 (bs, 1H, NH), 8.18 (s, 1H, H₈), 6.35 (s, 1H, H_{1'}), 5.51 (d, *J*=7.5 Hz, 1H, H_{3'}), 4.47-4.32 (m, 3H, H_{4'} and H_{5'}), 2.08 (s, 3H, CH₃ acetyl), 2.06 (s, 3H, CH₃ acetyl), 2.05 (s, 3H, CH₃ acetyl), 1.47 (s, 3H, 2'CCH₃).

Synthesis of 2-NH-benzyl-2'-C-methylguanosine 2',3',5'-tri-O-acetate (10.3).

To a solution of 2-bromo-2'-C-methylxanthosine 2',3',5'-tri-O-acetate (**10.2**, 0.35 g, 0.74 mmol) in dry ethanol (5 ml), benzylamine (0.53 ml, 4.74 mmol) was added and the reaction mixture was refluxed at 95 °C

for 16 h. After that time solvent was evaporated and crude residue was absorbed onto silica and purified on silica gel, using CHCl₃/MeOH (0 to 3%, gradient) as an eluent. The pure compound **10.3** was obtained as a yellowish solid (125 mg, 33%).

¹H NMR (500 MHz, DMSO) δ 10.68 (bs, 1H, NH), 7.79 (s, 1H, H₈), 7.36-7.25 (m, 5H, NHCH₂Ph), 6.69 (t, *J* = 6.0 Hz, 1H, NHCH₂Ph), 6.28 (s, 1H, H_{1'}), 5.73 (d, *J* = 8.0 Hz, 1H, H_{3'}), 4.62-4.50 (m, 2H, NHCH₂Ph), 4.30-4.24 (m, 3H, H_{4'} and H_{5'}), 2.11 (s, 3H, CH₃ acetyl), 2.06 (s, 3H, CH₃ acetyl), 2.00 (s, 3H, CH₃ acetyl), 1.36 (s, 3H, 2'CCH₃).

Synthesis of 2-NH-benzyl-2'-C-methylguanosine (10.4).

2-NH-benzyl-2'-C-methylguanosine 2',3',5'-tri-O-acetate (**10.3**, 125 mg, 0.24 mmol) was suspended in dry methanol (10 ml) in sealed tube and cooled down to 0 °C. After that solution was saturated with ammonia and

immediately sealed. Reaction mixture was allowed to warm to ambient temperature and reaction mixture was stirred overnight. After that time solvents were evaporated under reduced pressure and crude mixture was purified on silica gel, using CHCl₃/MeOH (8:2) as eluent. Pure compound **10.4** was obtained as white solid (70 mg, 74%).

¹H NMR (500 MHz, DMSO) δ 10.57 (bs, 1H, NH), 8.07 (s, 1H, H₈), 7.40 (d, *J* = 7.5 Hz, 2H, NHCH₂Ph), 7.35 (t, *J* = 7.5 Hz, 2H, NHCH₂Ph), 7.26 (t, *J* = 7.5 Hz, 1H, NHCH₂Ph), 6.89 (t, *J* = 5.5 Hz, 1H, NHCH₂Ph), 5.78 (s, 1H, H_{1'}), 5.19 (d, *J* = 7.0 Hz,

^1H , 3'OH), 5.09-5.07 (m, 3H, 5'OH and 2'OH), 4.50 (d, $J=5.5$ Hz, 2H, NHCH_2Ph), 3.99 (d, $J=8.0$ Hz, 1H, H_3'), 3.88-3.79 (m, 3H, H_4' and $\text{H}_{5'a}$), 3.66-3.64 (m, 1H, $\text{H}_{5'b}$), 0.79 (s, 3H, 2'CCH₃).

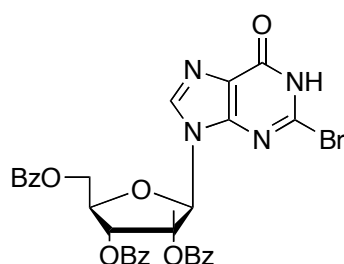
^{13}C NMR (126 MHz, DMSO) 156.71 (C6), 152.37 (C2), 150.20 (C4), 138.98 (ipso NHCH_2Ph), 128.35 (NHCH_2Ph), 127.82 (NHCH_2Ph), 127.02 (NHCH_2Ph), 116.63 (C5), 90.37 (C1'), 82.41 (C4'), 78.48 (C2'), 71.69 (C3'), 59.54 (C5'), 44.05 (NHCH_2Ph), 19.86 (2'CCH₃).

HPLC (System 1) $t_R=7.79$ min.

MS (ES+) m/z : 388.16 ($\text{M}+\text{H}^+$, 100%).

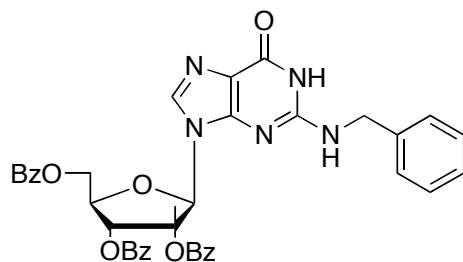
HRMS $\text{C}_{18}\text{H}_{21}\text{N}_5\text{O}_5$ Calculated: 387.1578 found: 387.1522.

Synthesis of 2-bromo-2'-*C*-methylxanthosine 2',3',5'-tri-*O*-benzoate (10.6).



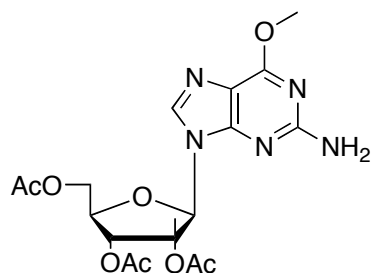
To a precooled (0 °C) solution of tetra-*O*-benzoyl-2'-*C*-methyl- β -D-ribofuranose (1.00g, 1.72 mmol), 2-bromohypoxanthine (0.41 g, 1.89 mmol) and DBU (0.77 ml, 5.10 mmol) in anhydrous acetonitrile (20 ml) under argon atmosphere, trimethylsilyl triflate (1.25 ml, 6.88 mmol) was added dropwise. The reaction mixture was heated at 65 °C for 8 h. After that time reaction mixture was allowed to cool down to ambient temperature and poured into saturated solution of sodium bicarbonate (150 ml), and extracted with DCM (3x 100 ml). The combined organic phases were dried over Na_2SO_4 and evaporated in vacuo. The residue was purified on silica gel, using $\text{CHCl}_3/\text{MeOH}$ (0 to 4%, gradient) as en eluent, to give pure product **10.6** as a yellowish solid (0.91 g, 78%).

^1H NMR (500 MHz, CDCl_3) δ 8.21-8.17 (m, 4H, Bn), 8.05 (s, 1H, H_8), 8.03–7.99 (m, 2H, Bn), 7.66–7.62 (m, 1H, Bn), 7.58-7.49 (m, 3H, Bn), 7.41-7.36 (m, 4H, Bn), 6.65 (s, 1H, H_1'), 5.95 (d, $J=6.5$ Hz, 1H, H_3'), 5.01-4.96 (m, 2H, H_5'), 4.72- 4.68 (m, 1H, H_4'), 1.62 (s, 3H, 2'CCH₃).

Synthesis of 2-NH-benzyl-2'-C-methylguanosine 2',3',5'-tri-O-benzoate (10.7).

To a solution of 2-bromo-2'-C-methylxanthosine 2',3',5'-tri-O-benzoate (**10.6**, 0.50 g, 0.74 mmol) in dry ethanol (5 ml), benzylamine (0.53 ml, 4.74 mmol) was added and the reaction mixture was refluxed at 95 °C for 16 h. After that time solvent was evaporated and crude residue was absorbed onto silica and purified on silica gel, using CHCl₃/MeOH (0 to 3%, gradient) as an eluent. The pure compound **10.7** was obtained as a yellowish solid (225 mg, 43%).

¹H NMR (500 MHz, CDCl₃) δ 8.23-8.20 (m, 2H, Bn), 8.07 (s, 1H, H₈), 8.05–7.98 (m, 4H, Bn), 7.68–7.64 (m, 2H, Bn), 7.61-7.57 (m, 2H, Bn), 7.53-7.38 (m, 9H, Bn and NHCH₂Ph), 6.62 (s, 1H, H_{1'}), 5.97 (d, *J* = 6.5 Hz, 1H, H_{3'}), 5.32-5.27 (m, 2H, NHCH₂Ph), 5.02-4.97 (m, 2H, H_{5'}), 4.72- 4.68 (m, 1H, H_{4'}), 1.60 (s, 3H, 2'CCH₃).

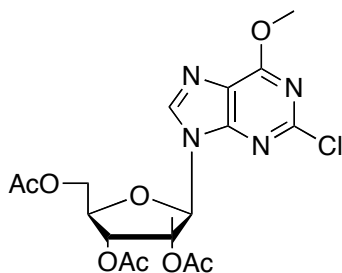
Synthesis of 2-amino-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine 2',3',5'-tri-O-acetate (10.10).

2-amino-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine (**5.4**, 2.00 g, 6.42 mmol) was suspended in dry acetonitrile (100 ml) and Et₃N (3.49 ml, 25.06 mmol) and DMAP (0.078 g, 0.64 mmol) were added under argon, followed by the dropwise addition of acetic anhydride (2.18 ml, 23.13 mmol). The reaction mixture was stirred at ambient temperature for 16 h. After that time reaction was concentrated to 1/3 volume and solids were filtered off washed with diethyl ether and dried. The pure compound **10.10** was obtained as a white solid (1.85g, 66%).

¹H NMR (500 MHz, CDCl₃) δ 7.75 (s, 1H, H₈), 6.35 (bs, 2H, NH₂), 6.16 (s, 1H, H_{1'}), 6.06 (d, *J* = 7.5 Hz, 1H, H_{3'}), 5.01 (bs, 2H, NH₂), 4.60 (dd, *J* = 4.0 Hz, *J* = 11.0 Hz, 1H,

H_{5'a}), 4.44-4.35 (m, 3H, H_{4'} and H_{5'b}), 4.09 (s, 3H, 6OCH₃), 2.18 (s, 3H, CH₃ acetyl), 2.16 (s, 3H, CH₃ acetyl), 2.12 (s, 3H, CH₃ acetyl), 1.42 (s, 3H, 2'CCH₃).

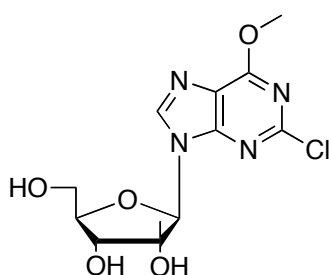
Synthesis of 2-chloro-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 2',3',5'-tri-*O*-acetate (10.11).



A solution of acetyl chloride (0.44 ml, 8.83 mmol) in dry DCM (22 ml) under argon atmosphere was chilled in NaCl/ice/H₂O bath for 15 min. Tetrabutylammonium nitrite (0.33 g, 1.14 mmol) was dissolved in dry DCM (4 ml) and this solution was added dropwise to the cold, stirred solution of AcCl/DCM. A solution of **10.10** (0.50 g, 1.14 mmol) in dry DCM (2 ml) was added dropwise to the cold solution and stirring was continued for 5 min. After completion of the reaction (as indicated by the TLC analysis), the reaction mixture was added dropwise at a rapid rate to a cold (ice/H₂O bath) vigorously stirred mixture of saturated NaHCO₃/H₂O/DCM. The layers were separated and the organic phase was dried over Na₂SO₄. Volatiles were evaporated and the residue was purified on silica gel, using CHCl₃/MeOH (0 to 2%, gradient) as an eluent. The pure product **10.11** was obtained as a white solid (0.32 g, 62%).

¹H NMR (500 MHz, DMSO) δ 8.56 (s, 1H, H₈), 6.47 (s, 1H, H_{1'}), 5.56 (d, *J*=6.5 Hz, 1H, H_{3'}), 4.45-4.33 (m, 3H, H_{4'} and H_{5'}), 4.13 (s, 3H, 6OCH₃), 2.13 (s, 3H, CH₃ acetyl), 2.10 (s, 3H, CH₃ acetyl), 2.06 (s, 3H, CH₃ acetyl), 1.36 (s, 3H, 2'CCH₃).

Synthesis of 2-chloro-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine (10.12).



2-chloro-6-*O*-methyl-9-(2'-*C*-methyl-β-D-ribofuranosyl) purine 2',3',5'-tri-*O*-acetate (**10.10**, 220 mg, 0.48 mmol) was suspended in dry methanol (15 ml) in sealed tube and cooled down to 0 °C. After that solution was saturated with ammonia and immediately sealed. Reaction mixture was allowed to warm to ambient temperature and reaction mixture was stirred overnight. After that time solvents were

evaporated under reduced pressure and crude mixture was purified on silica gel, using $\text{CHCl}_3/\text{MeOH}$ (9:1) as eluent. Pure compound **10.12** was obtained as white solid (143 mg, 90%).

^1H NMR (500 MHz, MeOD) δ 8.27 (s, 1H, H_8), 6.11 (s, 1H, $\text{H}_{1'}$), 4.27 (d, $J = 9.0$ Hz, 1H, $\text{H}_{3'}$), 4.23 (s, 3H, 6OCH_3), 4.12-4.03 (m, 2H, $\text{H}_{4'}$ and $\text{H}_{5'a}$), 3.91-3.89 (m, 1H, $\text{H}_{5'b}$), 0.96 (s, 3H, $2'\text{CCH}_3$).

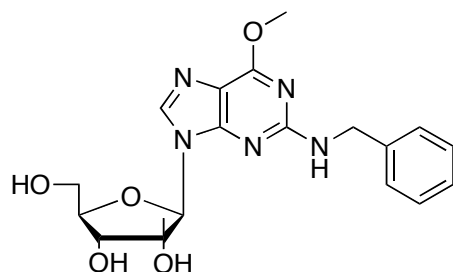
^{13}C NMR (126 MHz, MeOD) δ 162.61 (C6), 154.40 (C2), 153.83 (C4), 143.54 (C8), 121.15 (C5), 93.30 ($\text{C}_{1'}$), 84.55 ($\text{C}_{4'}$), 80.33 ($\text{C}_{2'}$), 73.32 ($\text{C}_{3'}$), 61.00 ($\text{C}_{5'}$), 55.69 (6OCH_3), 20.18 ($2'\text{CCH}_3$).

HPLC (System 1) $t_R = 7.35$ min.

HPLC (System 2) $t_R = 11.82$ min.

MS (ES+) m/z : 331.07 ($\text{M} + \text{H}^+$, 100%).

Synthesis of 2-NH-benzyl-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine (**10.13**).



To a solution of 2-chloro-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine (**10.12**, 0.50 g, 1.33 mmol) in dry ethanol (5 ml), benzylamine (0.73 ml, 6.66 mmol) was added and the reaction mixture was refluxed at 95°C for 16 h. After that time solvent was evaporated

and crude residue was absorbed onto silica and purified on silica gel, using $\text{CHCl}_3/\text{MeOH}$ (0 to 3%, gradient) as an eluent. The pure compound **10.13** was obtained as a yellowish solid (50 mg, 9%).

^1H NMR (500 MHz, MeOD) δ 8.30 (s, 1H, H_8), 7.38 (d, $J = 7.5$ Hz, 2H, NHCH_2Ph), 7.28 (t, $J = 7.5$ Hz, 2H, NHCH_2Ph), 7.19 (d, $J = 7.5$ Hz, 1H, NHCH_2Ph), 6.03 (s, 1H, $\text{H}_{1'}$), 4.61 (d, $J = 4.0$ Hz, 2H, NHCH_2Ph), 4.23 (d, $J = 9.0$ Hz, 1H, $\text{H}_{3'}$), 4.06-3.98 (m, 2H, $\text{H}_{4'}$ and $\text{H}_{5'a}$), 4.02 (s, 3H, 6OCH_3), 3.85 (dd, $J = 4.0$ Hz, $J = 11.0$ Hz, 1H, $\text{H}_{5'b}$), 0.93 (s, 3H, $2'\text{CCH}_3$).

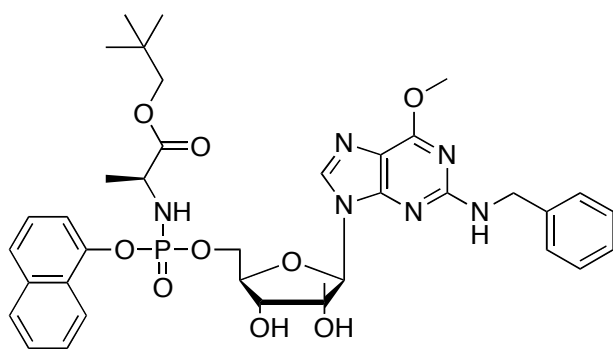
^{13}C NMR (126 MHz, MeOD) δ 162.42 (C6), 160.91 (C2), 154.67 (C4), 141.62 (ipso NHCH_2Ph), 139.19 (C8), 129.37, 128.54, 127.81 (NHCH_2Ph), 115.17 (C5), 92.79 (C1'), 84.13 (C4'), 80.37 (C2'), 73.67 (C3'), 61.35 (C5'), 54.14 (6OCH_3), 20.27 ($2'\text{CCH}_3$).

HPLC (System 1) t_{R} = 7.35 min.

HPLC (System 2) t_{R} = 11.82 min.

MS (ES+) m/z : 402.17 ($\text{M}+\text{H}^+$, 100%).

Synthesis of 2-*NH*-benzyl-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.16a).



Prepared according to the Standard Procedure 7 from: 2-*NH*-benzyl-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**10.13**, 100 mg, 0.25 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 190

mg, 0.50 mmol), and *t*BuMgCl (0.50 ml, 0.50 mmol) in 3 ml of dry THF. The crude mixture was purified by column chromatography, using $\text{CHCl}_3/\text{MeOH}$ (0 to 5%, gradient) as en eluent. To give a pure product **10.16a** as white foam (36 mg, 19%).

^{31}P NMR (202 MHz, MeOD) δ 4.09, 3.95.

^1H NMR (500 MHz, MeOD) δ 8.19-8.16 (m, 1H, $\text{H}_8\text{-naph}$), 7.96, 7.95 (2s, 1H, H_8), 7.93-7.87 (m, 1H, $\text{H}_5\text{-Naph}$), 7.67-7.64 (m, 1H, $\text{H}_4\text{-Naph}$), 7.55-7.47 (m, 3H, H_2 , H_7 , $\text{H}_6\text{-Naph}$), 7.42-7.19 (m, 6H, $\text{H}_3\text{-Naph}$ and NHCH_2Ph), 5.99, 5.98 (2s, 1H, $\text{H}_{1'}$), 4.62-4.51 (m, 4H, $\text{H}_{5'}$ and NHCH_2Ph), 4.42-4.27 (m, 2H, $\text{H}_{3'}$, $\text{H}_{4'}$), 4.04-3.99 (m, 1H, CHCH_3), 4.01 (s, 3H, 6OCH_3), 3.74, 3.71, 3.63, 3.57 (2AB, J_{AB} = 10.5 Hz, 2H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$), 1.34 (d, J = 7.5 Hz, 3H, CHCH_3), 0.97, 0.95 (2s, 3H, $2'\text{CCH}_3$), 0.84, 0.82 (2s, 9H, $\text{OCH}_2\text{C}(\text{CH}_3)_3$).

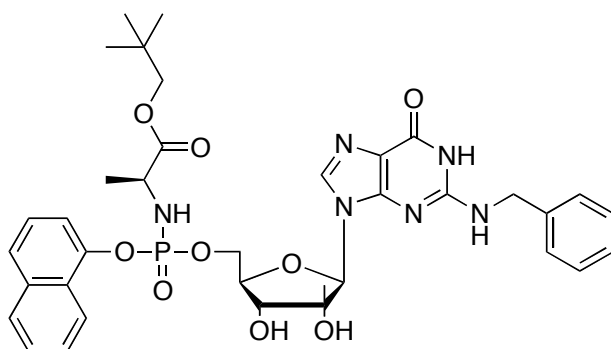
^{13}C NMR (126 MHz, MeOD) δ 175.00, 174.78 (2d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, C=O), 162.46 (C6), 160.89 (C2), 154.45 (C4), 148.00, 147.96 (d, $^2J_{\text{C-O-P}}$ = 3.8 Hz, ipso Naph), 141.22, 140.98 (ipso NHCH_2Ph), 139.55, 139.33 (C8), 136.28, 136.25 (C10-Naph),

129.59, 129.49, 129.42, 129.39, 129.37, 128.83, 128.77, 128.65, 128.46, 128.40, 128.32, 128.15 (CH-Naph and NHCH₂Ph), 127.86, 127.82 (2d, $^3J_{C-C-O-P}$ = 6.3 Hz, C9-Naph), 127.73, 127.69, 127.44, 127.41, 126.49, 126.46, 125.92, 125.89, 122.77, 122.74 (CH-Naph), 116.20, 116.21 (C2-Naph), 115.62 (C5), 93.60, 93.04 (C1'), 82.31, 82.17 (2d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.97, 79.90 (C2'), 75.33, 75.21 (OCH₂C(CH₃)₃), 74.97, 74.76 (C3'), 68.39, 67.97 (2d, $^2J_{C-O-P}$ = 5.0 Hz, C5'), 54.12 (6OCH₃), 51.73, 51.66 (CHCH₃), 46.56, 46.50 ((NHCH₂Ph), 32.31, 32.23 (OCH₂C(CH₃)₃), 26.74, 26.71 (OCH₂C(CH₃)₃), 20.81 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.62 (d, $^3J_{C-C-N-P}$ = 7.5 Hz, CHCH₃), 20.42, 20.20 (2'CCH₃).

HPLC (System 1) t_R = 22.95, 23.48 min.

HRMS calculated for C₃₇H₄₅N₆O₉PNa: 771.2987; found 771.2965.

Synthesis of 2-NH-benzyl-2'-C-methylguanosine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.8a).



Prepared according to the Standard Procedure 14, from: **10.16a** (36 mg, 0.048 mmol) was dissolved in dry acetonitrile (3 ml) under argon atmosphere and NaI (8.6 mg, 0.058 mmol) was added, followed by the

addition of trimethylsilyl chloride (0.007 ml, 0.058 mmol). The reaction mixture was stirred at ambient temperature for 4 h. After work up, the residue was purified by preparative TLC, using CHCl₃/MeOH (96:4) as an eluent. The pure compound **10.8a** was obtained as off white solid (10.2 mg, 29%).

^{31}P NMR (202 MHz, MeOD) δ 4.08, 3.99.

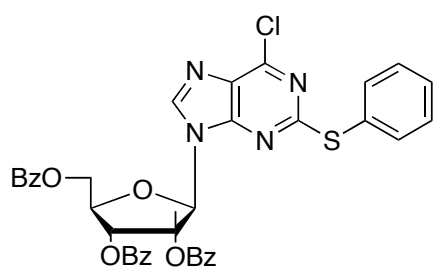
^1H NMR (500 MHz, MeOD) δ 8.18-8.16 (m, 1H, H₈-naph), 7.91-7.85 (m, 2H, H₅-Naph and H₈), 7.71, 7.68 (2d, J = 7.0 Hz, 1H, H₄-Naph), 7.57-7.43 (m, 3H, H₂, H₇, H₆-Naph), 7.39-7.23 (m, 6H, H₃-Naph and NHCH₂Ph), 5.57 (s, 1H, H_{1'}), 4.59-4.47 (m, 4H, H_{5'} and NHCH₂Ph), 4.28-4.21 (m, 2H, H_{3'}, H_{4'}), 4.04-3.99 (m, 1H, CHCH₃), 3.75, 3.72, 3.64, 3.58 (2AB, J_{AB} = 10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.39, 1.33 (2d, J = 7.5 Hz, 3H, CHCH₃), 0.99, 0.97 (2s, 3H, 2'CCH₃), 0.83, 0.82 (2s, 9H, OCH₂C(CH₃)₃).

^{13}C NMR (126 MHz, MeOD) δ 174.97, 174.95 (C=O), 159.40 (C6), 159.22 (C2), 153.92 (C4), 147.87, 147.58 (ipso Naph), 141.20, 140.73 (ipso NHCH_2Ph), 139.74 (C8), 136.26, 136.24 (C10-Naph), 129.69, 129.65, 129.40, 129.37, 128.85, 128.78, 128.74, 128.43, 128.40 (CH-Naph and NHCH_2Ph), 127.89, 127.83 (2d, $^3J_{\text{C-C-O-P}} = 6.3$ Hz, C9-Naph), 127.74, 127.69, 127.46, 126.49, 126.46, 125.92, 125.89, 122.77, 122.74 (CH-Naph), 118.10 (C5), 116.16, 116.13 (C2-Naph), 93.36 (C1'), 82.04, 81.98 (2d, $^3J_{\text{C-C-O-P}} = 8.8$ Hz, C4'), 79.95, 79.89 (C2'), 75.35, 75.32 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 74.69, 74.46 (C3'), 68.20, 67.70 (2d, $^2J_{\text{C-O-P}} = 5.0$ Hz, C5'), 51.78, 51.68 (CHCH_3), 46.11, 46.07 ((NHCH_2Ph)), 32.25 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 26.69 ($\text{OCH}_2\text{C}(\text{CH}_3)_3$), 20.82, 20.59 (2d, $^3J_{\text{C-C-N-P}} = 6.3$ Hz, CHCH_3), 20.29 ($2'\text{CCH}_3$).

HPLC (System 1) $t_{\text{R}} = 18.71, 19.03$ min.

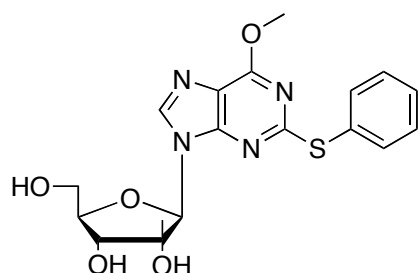
HRMS calculated for $\text{C}_{36}\text{H}_{44}\text{N}_6\text{O}_9\text{P}$: 734.2852; found 734.2837.

Synthesis of 2-C-thiophenyl-6-C-chloro-9-(2'-C-methyl- β -D-ribofuranosyl) purine 2',3',5'-tri-O-benzoate (10.17).



A mixture of 2-amino-6-chloro-9-(2-C-methyl-2,3,5-tri-O-benzoyl- β -D-ribofuranosyl)purine (**5.2**, 1.00 g, 1.61 mmol), diphenyl disulfide (1.76 g, 8.04 mmol) and isopentyl nitrite (2.13 ml, 16.17 mmol) was suspended in dry acetonitrile under argon atmosphere and refluxed at 100 °C, for 3 h. After that time solvent was evaporated and the residue was purified by flash chromatography, using $\text{CHCl}_3/\text{MeOH}$ (98:2) as eluent. Isolated fractions (1.30 g) containing the desired product were contaminated with some impurities related to isopentyl nitrite, having same R_f as the desired product.

Synthesis of 2-*C*-thiophenyl-6-*O*-methyl-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**10.18**).



To a solution of 2-*C*-thiophenyl-6-*C*-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine (**10.17**, 1.30 g, 1.80 mmol) in anhydrous methanol (10 ml) at 0 °C, NaOMe (0.59 g, 10.82 mmol) was added. The mixture was stirred at room temperature for 16 hours and then quenched by addition of amberlite (H⁺). The mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 96:4) to give the pure compound **10.18** (287 mg, 44%) as a white solid.

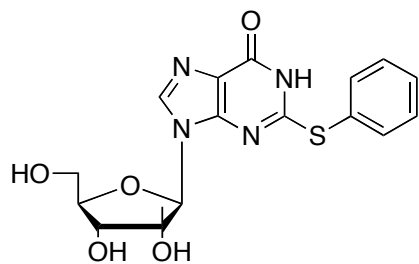
¹H NMR (500 MHz, MeOD) δ 8.58 (s, 1H, H₈), 7.67-7.65 (m, 2H, *SPh*), 7.47-7.45 (m, 3H, *SPh*), 6.00 (s, 1H, H_{1'}), 4.10 (d, *J*=8.0 Hz, 1H, H_{3'}), 4.04-4.01 (m, 1H, H_{4'}), 3.95-3.93 (m, 1H, H_{5'a}), 3.88 (s, 3H, 6OCH₃), 3.80-3.77 (m, 1H, H_{5'b}), 0.93 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 166.69 (C6), 161.42 (C2), 153.64 (C4), 142.30 (C8), 136.69 (CH *SPh*), 131.51 (ipso *SPh*), 130.30, 130.12 (CH *SPh*), 119.68 (C5), 93.32 (C1'), 84.40 (C4'), 80.24 (C2'), 73.54 (C3'), 61.43 (C5'), 54.76 (6OCH₃), 20.23 (2'CCH₃).

HPLC (System 1) *t*_R = 13.44 min.

HRMS calculated for C₁₈H₂₀N₄O₅SNa: 427.1149; found 427.1136

Synthesis of 2-*C*-thiophenyl-2'-*C*-methylguanosine (**10.19**).



2-*C*-thiophenyl-6-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine (**10.18**, 270 mg, 0.67 mmol) was dissolved in dry acetonitrile (10 ml) under argon atmosphere and NaI (109 mg, 0.73 mmol) was added, followed by the addition of trimethylsilyl chloride (0.09 ml, 0.73 mmol). The reaction mixture was stirred at reflux for 4 h. After that time reaction mixture was allowed to cool to room

temperature and poured into saturated solution of sodium bicarbonate (50 ml) and extracted with DCM (3x 10ml). The combined organic phases were dried over Na₂SO₄ and evaporated to dryness. The residue was purified by column chromatography using CHCl₃/MeOH (9:1) as an eluent. The pure compound **10.19** was obtained as off white solid (110 mg, 42%).

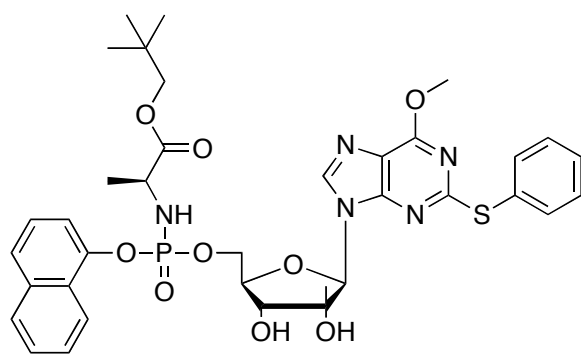
¹H NMR (500 MHz, MeOD) δ 8.39 (s, 1H, H₈), 7.66-7.42 (m, 2H, *SPh*), 7.47-7.45 (m, 3H, *SPh*), 6.00 (s, 1H, H_{1'}), 3.93 (app bs, 2H, H_{3'} and H_{4'}), 3.87-3.67 (m, 2H, H_{5'}), 0.86 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 159.23 (C6), 158.83 (C2), 149.85 (C4), 140.02 (C8), 136.68, 131.38, 130.76 (CH *SPh*), 128.27 (ipso *SPh*), 122.68 (C5), 93.12 (C1'), 84.25 (C4'), 80.16 (C2'), 73.38 (C3'), 61.46 (C5'), 20.19 (2'CCH₃).

HPLC (System 1) t_R = 9.18 min.

HRMS calculated for C₁₇H₁₈N₄O₅S: 390.1077; found 390.1051

Synthesis of 2-*C*-thiophenyl-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine 5'-*O*-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.20a**).**



Prepared according to the Standard Procedure 7 from: 2-*C*-thiophenyl-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**10.18**, 250 mg, 0.62 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 409 mg,

1.24 mmol), and *t*BuMgCl (1.24 ml, 1.24 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as an eluent. To give a pure product **10.20a** as white foam (97 mg, 21%).

³¹P NMR (202 MHz, MeOD) δ 3.76, 3.38.

¹H NMR (500 MHz, MeOD) δ 8.23, 8.21 (2s, 1H, H₈), 8.15, 7.98 (2d, *J*=8.5 Hz, 1H, H₈-Naph), 7.86, 7.82 (2d, *J*=8.0 Hz, 1H, H₅-Naph), 7.67-7.62 (m, 2H, H₄-Naph and *SPh*), 7.52-7.42 (m, 7H, H₇, H₆, H₂-Naph and *SPh*), 7.34-7.30 (m, 1H, H₃-Naph),

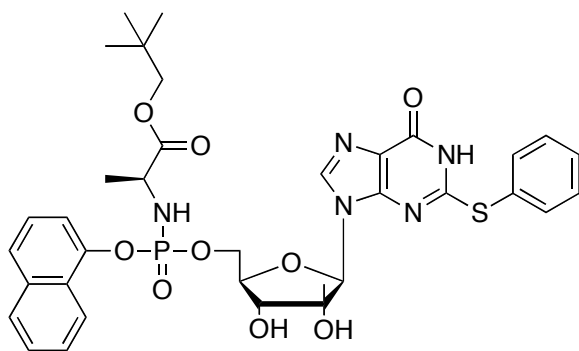
5.95, 5.93 (2s, 1H, H_{1'}), 4.36-4.22 (m, 2H, H_{5'}), 4.20-4.10 (m, 1H, H_{4'}), 4.03-3.98 (m, 2H, H_{3'} and CHCH₃), 3.92, 3.89 (2s, 3H, 6OCH₃), 3.72, 3.67, 3.58, 3.56 (2AB, J_{AB} =10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.31, 1.30 (2d, J =7.5 Hz, 3H, CHCH₃), 0.96, 0.94 (2s, 3H, 2'CCH₃), 0.85, 0.84 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 174.89, 174.69 (C=O), 167.05, 166.96 (C6), 161.46, 161.43 (C2), 153.15 (C4), 147.96 (d, $^2J_{C-O-P}$ = 7.5 Hz, ipso Naph), 147.89 (d, $^2J_{C-O-P}$ = 6.3 Hz, ipso Naph), 143.14, 142.92 (C8), 136.95, 136.84 (CH SPh), 136.23, 136.19 (C10-Naph), 131.05 (ipso SPh), 130.58, 130.46, 130.35 (CH SPh), 128.82, 128.73 (CH Naph), 127.78, 127.70 (C9-Naph), 127.59, 127.40, 127.24, 126.41, 125.81, 125.71, 122.75, 122.63 (CH Naph), 120.31 (C5), 116.13 (d, $^3J_{C-C-O-P}$ = 3.8 Hz, C2-Naph), 115.86 (d, $^3J_{C-C-O-P}$ = 2.5 Hz, C2-Naph), 94.66 (C1'), 82.58 (d, $^3J_{C-C-O-P}$ = 7.5 Hz, C4'), 82.52 (d, $^3J_{C-C-O-P}$ = 8.8 Hz, C4'), 79.81, 79.72 (C2'), 75.29 (OCH₂C(CH₃)₃), 74.93, 74.88 (C3'), 68.89, 68.62 (C5'), 54.79, 54.76 (6OCH₃), 51.70, 51.60 (CHCH₃), 32.22 (OCH₂C(CH₃)₃), 26.67 (OCH₂C(CH₃)₃), 20.86 (d, $^3J_{C-C-N-P}$ = 6.3 Hz, CHCH₃), 20.66 (d, $^3J_{C-C-N-P}$ = 7.5 Hz, CHCH₃), 20.27, 20.23 (2'CCH₃).

HPLC (System 1) t_R = 24.96, 25.57 min.

HRMS calculated for C₃₆H₄₂N₅O₉PS: 751.2471; found 751.2470

Synthesis of 2-C-thiophenyl-2'-C-methylguanosine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.21a).



Prepared according to the Standard Procedure 7 from: 2-C-thiophenyl-2'-C-methylguanosine (**10.19**, 110 mg, 0.28 mmol), α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 187 mg, 0.56 mmol), and *t*BuMgCl (0.56 ml,

0.56 mmol) in 3 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 3%, gradient) as an eluent. To give the pure product **10.20a** as white foam (45 mg, 21%).

³¹P NMR (202 MHz, MeOD) δ 3.74, 3.32.

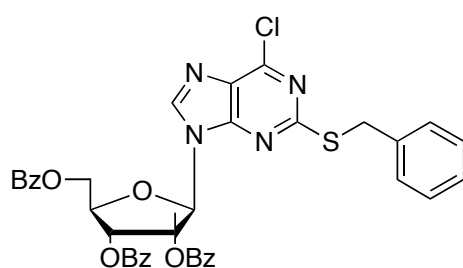
^1H NMR (500 MHz, MeOD) δ 8.18-8.16, 8.04-8.03 (2m, 1H, H₈-Naph), 8.01, 7.91 (2s, 1H, H₈), 7.89-7.84 (m, 1H, H₅-Naph), 7.69-7.65 (m, 3H, H₄-Naph and *SPh*), 7.56-7.45 (m, 6H, H₇, H₆, H₂-Naph and *SPh*), 7.37-7.33 (m, 1H, H₃-Naph), 5.75, 5.74 (2s, 1H, H_{1'}), 4.06-3.97 (m, 4H, H_{5'}, H_{4'} and H_{3'}), 3.76-3.70, 3.61-3.56 (2m, 3H, OCH₂C(CH₃)₃ and CHCH₃), 1.33, 1.32 (2d, $J=7.0$ Hz, 3H, CHCH₃), 0.92, 0.90 (2s, 3H, 2'CCH₃), 0.87, 0.86 (2s, 9H, OCH₂C(CH₃)₃).

^{13}C NMR (126 MHz, MeOD) δ 174.70, 174.42 (C=O), 166.12, 165.68 (C6), 162.07, 161.55 (C2), 157.26, 157.00 (C4), 148.43, 147.99 (ipso Naph), 141.11, 141.08 (C8), 137.16, 137.00 (CH *SPh*), 136.25 (C10-Naph), 132.40 (ipso *SPh*), 131.75, 131.65, 131.16, 131.09, 128.87, 128.80 (CH Naph and CH *SPh*), 127.78, 127.70 (C9-Naph), 127.43, 127.27, 126.39, 125.86, 125.76, 122.72, 122.68 (CH Naph), 116.14, 116.11 (C2-Naph), 114.34 (C5), 94.85 (C1'), 82.29, 82.23 (C4'), 79.50 (C2'), 75.30 (OCH₂C(CH₃)₃), 74.70, 74.63 (C3'), 69.17, 69.14 (C5'), 54.79, 54.76 (6OCH₃), 51.72, 51.57 (CHCH₃), 32.26 (OCH₂C(CH₃)₃), 26.71, 26.69 (OCH₂C(CH₃)₃), 20.83 (d, $^3J_{\text{C-C-N-P}} = 5.0$ Hz, CHCH₃), 20.63 (d, $^3J_{\text{C-C-N-P}} = 7.5$ Hz, CHCH₃), 20.27 (2'CCH₃).

HPLC (System 1) $t_R = 20.71, 21.03$ min.

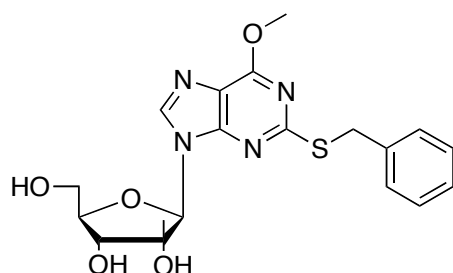
HRMS calculated for C₃₅H₄₅N₅O₉PSNa: 760.2254; found 760.2261.

Synthesis of 2-*C*-thiobenzyl-6-*C*-chloro-9-(2'-*C*-methyl- β -D-ribofuranosyl) purine 2',3',5'-tri-*O*-benzoate (10.22).



A mixture of 2-amino-6-chloro-9-(2-*C*-methyl-2,3,5-tri-*O*-benzoyl- β -D-ribofuranosyl)purine (**5.2**, 1.00 g, 1.61 mmol), dibenzyl disulfide (1.98 g, 8.04 mmol) and isopentyl nitrite (2.13 ml, 16.17 mmol) was suspended in dry acetonitrile under argon atmosphere and refluxed at 100 °C, for 3 h. After that time solvent was evaporated and the residue was purified by flash chromatography, using CHCl₃/MeOH (98:2) as an eluent. Isolated fractions (0.70 g) containing the desired product were contaminated with some impurities related to isopentyl nitrite, having same R_f as the desired product.

Synthesis of 2-*C*-thiobenzyl-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**10.23**).



To a solution of 2-*C*-thiophenyl-6-*C*-chloro-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-*D*-ribofuranosyl)purine (**10.22**, 0.70 g, 0.95 mmol) in anhydrous methanol (10 ml) at 0 °C, NaOMe (0.26 g, 5.71 mmol) was added. The mixture was stirred at room temperature for 16 hours and then quenched by addition of amberlite (H⁺). The mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH, 96:4) to give the pure compound **10.23** (220 mg, 33%) as a white solid.

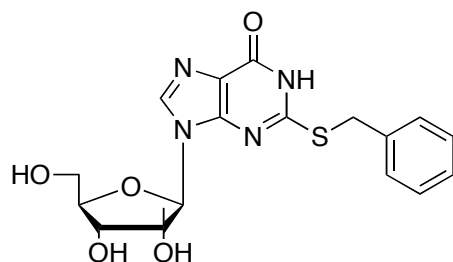
¹H NMR (500 MHz, MeOD) δ 8.64 (s, 1H, H₈), 7.49-7.48 (m, 2H, SCH₂Ph), 7.32-7.29 (m, 2H, SCH₂Ph), 7.23-7.20 (m, 1H, SCH₂Ph), 6.18 (s, 1H, H_{1'}), 4.49, 4.45 (AB, *J*= 10.0 Hz, 2H, SCH₂Ph), 4.21 (d, *J*=9.0 Hz, H_{3'}), 4.12 (s, 3H, 6OCH₃), 4.10-4.04 (m, 2H, H_{4'} and H_{5'a}), 3.90-3.87 (m, 1H, H_{5'b}), 0.93 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 166.55 (C6), 161.48 (C2), 153.58 (C4), 141.65 (C8), 139.35 (ipso SCH₂Ph), 130.09, 139.51, 128.11 (CH SCH₂Ph), 119.19 (C5), 92.91 (C1'), 84.32 (C4'), 80.43 (C2'), 73.32 (C3'), 60.97 (C5'), 55.00 (6OCH₃), 36.58 (SCH₂Ph), 20.30 (2'CCH₃).

HPLC (System 1) t_R = 14.12 min.

HRMS calculated for C₁₉H₂₂N₄O₅S: 418.1321; found 418.1317.

Synthesis of 2-*C*-thiobenzyl-2'-*C*-methylguanosine (**10.24**).



2-*C*-thiobenzyl-6-*C*-chloro-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (**10.23**, 400 mg, 0.96 mmol) was dissolved in dry acetonitrile (20 ml) under argon atmosphere and NaI (160 mg, 1.05 mmol) was added, followed by the addition of trimethylsilyl chloride (0.13 ml, 1.05 mmol). The reaction mixture was stirred at

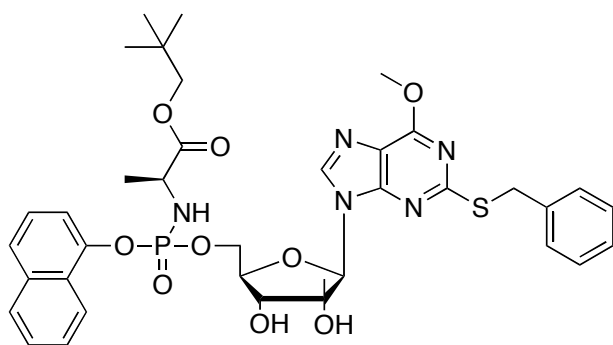
reflux for 4 h. After that time reaction mixture was allowed to cool to room temperature and poured into saturated solution of sodium bicarbonate (50 ml) and extracted with DCM (3x 10ml). The combined organic phases were dried over Na₂SO₄ and evaporated to dryness. The residue was purified by column chromatography using CHCl₃/MeOH (9:1) as an eluent. The pure compound **10.19** was obtained as off white solid (202 mg, 52%).

¹H NMR (500 MHz, MeOD) δ 8.69 (s, 1H, H₈), 7.49 (d, *J*= 7.5 Hz, 2H, SCH₂Ph), 7.33 (t, *J*= 7.5 Hz, 2H, SCH₂Ph), 7.23 (d, *J*= 7.5 Hz, 1H, SCH₂Ph), 6.12 (s, 1H, H_{1'}), 4.52, 4.48 (AB, *J*= 10.0 Hz, 2H, SCH₂Ph), 4.18 (d, *J*=9.0 Hz, H_{3'}), 4.10-4.02 (m, 2H, H_{4'} and H_{5'a}), 3.89-3.87 (m, 1H, H_{5'b}), 0.92 (s, 3H, 2'CCH₃).

¹³C NMR (126 MHz, MeOD) δ 159.98 (C6), 159.16 (C2), 149.98 (C4), 139.19 (C8), 138.09 (ipso SCH₂Ph), 130.38, 129.70, 128.58 (CH SCH₂Ph), 121.96 (C5), 92.62 (C1'), 84.24 (C4'), 80.52 (C2'), 73.19 (C3'), 60.81 (C5'), 36.06 (SCH₂Ph), 20.39 (2'CCH₃).

HPLC (System 1) t_R = 11.20 min.

Synthesis of 2-C-thiobenzyl-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine 5'-O-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.25a).



Prepared according to the Standard Procedure 7 from: 2-C-thiobenzyl-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine (**10.23**, 220 mg, 0.53 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 350

mg, 1.05 mmol), and *t*BuMgCl (1.05 ml, 1.05 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as en eluent. To give a pure product **10.25a** as white foam (102 mg, 25%).

³¹P NMR (202 MHz, MeOD) δ 4.10, 3.90.

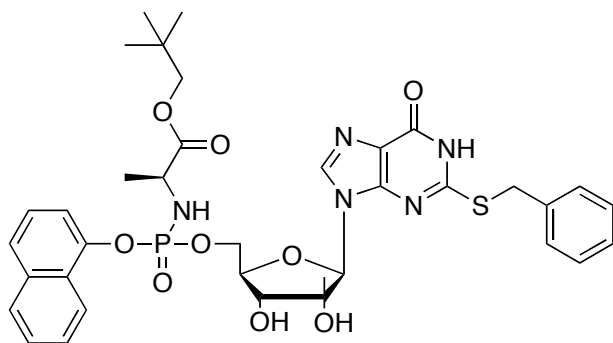
^1H NMR (500 MHz, MeOD) δ 8.26, 8.24 (2s, 1H, H₈), 8.18-8.13 (m, 1H, H₈-Naph), 7.84-7.90 (m, 1H, H₅-Naph), 7.68-7.61 (m, 2H, H₄-Naph), 7.56-7.40 (m, 5H, H₇, H₆, H₂-Naph and SCH₂Ph), 7.36-7.32 (m, 1H, H₃-Naph), 7.29-7.17 (m, 3H, SCH₂Ph), 6.17, 6.16 (2s, 1H, H_{1'}), 4.67-4.58 (m, 2H, H_{5'}), 4.46-4.25 (m, 4H, H_{4'}, H_{3'} and SCH₂Ph), 4.11-4.04 (m, 1H, CHCH₃), 4.07, 4.06 (2s, 3H, 6OCH₃), 3.74, 3.71, 3.61, 3.57 (2AB, J_{AB} =10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.34, 1.32 (2d, J =7.0 Hz, 3H, CHCH₃), 0.95, 0.93 (2s, 3H, 2'CCH₃), 0.84, 0.82 (2s, 9H, OCH₂C(CH₃)₃).

^{13}C NMR (126 MHz, MeOD) δ 175.02, 174.79 (2d, $^3J_{\text{C-C-O-P}}$ =5.0 Hz, C=O), 166.60, 166.59 (C6), 161.49 (C2), 153.41, 153.40 (C4), 147.99 (d, $^2J_{\text{C-O-P}}$ = 7.5 Hz, ipso Naph), 147.94 (d, $^2J_{\text{C-O-P}}$ = 6.3 Hz, ipso Naph), 141.84, 141.56 (C8), 139.11 (ipso SCH₂Ph), 136.30, 136.24 (C10-Naph), 130.14, 130.12, 129.57, 129.54, 128.19, 128.16 (CH Naph and SCH₂Ph), 127.86, 127.81 (C9-Naph), 127.73, 127.48, 127.47, 127.44, 126.60, 126.51, 125.94, 125.87, 122.80, 122.74 (CH Naph), 119.61 (C5), 116.47 (d, $^3J_{\text{C-C-O-P}}$ = 2.5 Hz, C2-Naph), 116.16 (d, $^3J_{\text{C-C-O-P}}$ = 3.8 Hz, C2-Naph), 93.55, 93.47 (C1'), 82.37, 82.30 (2d, $^3J_{\text{C-C-O-P}}$ = 8.8 Hz, C4'), 80.06, 79.98 (C2'), 75.47, 75.39 (OCH₂C(CH₃)₃), 74.74, 74.52 (C3'), 67.94 (d, $^2J_{\text{C-O-P}}$ = 5.0 Hz, C-5'), 67.54 (d, $^2J_{\text{C-O-P}}$ = 3.8 Hz, C-5'), 55.08 (6OCH₃), 51.83, 51.74 (CHCH₃), 36.70, 36.68 (SCH₂Ph), 32.28, 32.25 (OCH₂C(CH₃)₃), 26.81, 26.76 (OCH₂C(CH₃)₃), 20.97 (d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, CHCH₃), 20.74 (d, $^3J_{\text{C-C-N-P}}$ = 7.5 Hz, CHCH₃), 20.45, 20.42 (2'CCH₃).

HPLC (System 1) t_{R} = 25.62, 26.02 min.

HRMS calculated for C₃₇H₄₄N₅O₉PSNa: 788.2532; found 788.2513.

Synthesis of 2-C-thiophenyl-2'-C-methylguanosine 5'-O-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.26a).



Prepared according to the Standard Procedure 14, from: **10.25a** (36 mg, 0.047 mmol) was dissolved in dry acetonitrile (3 ml) under argon atmosphere and NaI (7.7 mg, 0.052 mmol) was added, followed by the

addition of trimethylsilyl chloride (0.007 ml, 0.052 mmol). The reaction mixture was stirred at ambient temperature for 4 h. After work up, the residue was purified by

preparative TLC, using CHCl₃/MeOH (96:4) as an eluent. The pure compound **10.26a** was obtained as off white solid (12 mg, 34%).

³¹P NMR (202 MHz, MeOD) δ 4.32, 4.29.

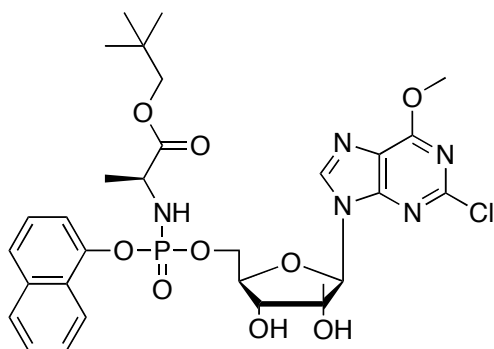
¹H NMR (500 MHz, MeOD) δ 8.22-8.19 (m, 1H, H₈-Naph), 8.11, 8.09 (2s, 1H, H₈), 7.91-7.86 (m, 1H, H₅-Naph), 7.72, 7.70 (2d, *J*=8.5 Hz, H₄-Naph), 7.55-7.49 (m, 5H, H₇, H₆, H₂-Naph and SCH₂Ph), 7.43-7.40 (m, 1H, H₃-Naph), 7.36-7.32 (m, 2H, CH SCH₂Ph), 7.28-7.25 (m, 1H, SCH₂Ph), 6.17, 6.16 (2s, 1H, H_{1'}), 4.68-4.55 (m, 4H, H_{5'} and SCH₂Ph), 4.30-4.25 (m, 1H, H_{4'}), 4.15-4.05 (m, 2H, H_{3'} and CHCH₃), 3.77 3.767, 3.75, 3.66, 3.61 (2AB, *J*_{AB}=10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.38, 1.34 (2d, *J*=7.0 Hz, 3H, CHCH₃), 0.98, 0.95 (2s, 3H, 2'CCH₃), 0.88, 0.87 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.04, 174.80 (C=O), 159.38 (C-6), 159.21 (C2), 149.95 (C4), 147.99, 147.93 (ipso Naph), 139.04, 138.84 (C8), 137.89 (ipso SCH₂Ph), 136.32, 136.30 (C10-Naph), 130.41, 130.40, 129.72, 129.70 (SCH₂Ph), 128.87, 128.81, 128.63, 128.61 (CH Naph), 127.87, 127.76 (C9-Naph), 127.56, 127.51, 126.49, 126.00, 122.83, 122.69 (CH Naph), 122.37, 122.33 (C5), 116.19 (C2-Naph), 93.08, 93.03 (C1'), 82.02, 81.93 (2d, ³*J*_{C-C-O-P}=8.8 Hz, C4'), 80.03, 79.95 (C2'), 75.37, 75.35 (OCH₂C(CH₃)₃), 74.40, 74.11 (C3'), 67.56 (d, ²*J*_{C-O-P}= 6.3 Hz, C5'), 67.00 (d, ²*J*_{C-O-P}=5.0 Hz, C5'), 51.83, 51.73 (CHCH₃), 36.16, 36.14 (SCH₂Ph), 32.26, 32.22 (OCH₂C(CH₃)₃), 26.67 (OCH₂C(CH₃)₃), 20.84 (d, ³*J*_{C-C-N-P}= 5.0 Hz, CHCH₃), 20.55 (d, ³*J*_{C-C-N-P}= 6.3 Hz, CHCH₃), 20.41 (2'CCH₃).

HPLC (System 1) t_R = 21.02, 21.24 min.

MS (ES+) m/z: 751.23 (MH⁺, 100%).

Synthesis of 2-C-chloro-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine 5'-O-[α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.27a).



Prepared according to the Standard Procedure 7 from: 2-C-chloro-6-O-methyl-9-(2'-C-methyl-β-D-ribofuranosyl) purine (**10.12**, 300 mg, 0.91 mmol), α-naphthyl-(2,2-dimethylpropoxy-L-alaninyl) phosphorochloridate (**3.3e**, 600 mg, 1.81

mmol), and *t*BuMgCl (1.81 ml, 1.81 mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using CHCl₃/MeOH (0 to 5%, gradient) as eluent. To give a pure product **10.25a** as white foam (78 mg, 13%).

³¹P NMR (202 MHz, MeOD) δ 4.04, 3.98.

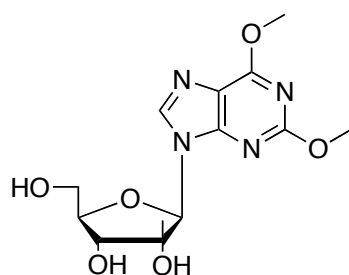
¹H NMR (500 MHz, MeOD) δ 8.40, 8.38 (2s, 1H, H₈), 8.18-8.10 (m, 1H, H₈-Naph), 7.85, 7.82 (2d, *J*=8.0 Hz, 2H, H₅-Naph), 7.66, 7.63 (2d, *J*=7.5 Hz, 1H, H₄-Naph), 7.52-7.41 (m, 3H, H₇, H₆, H₂-Naph), 7.37, 7.36 (2t, *J*=8.0 Hz, 1H, H₃-Naph), 6.11, 6.10 (2s, 1H, H₁'), 4.73-4.58 (m, 2H, H₅'), 4.35-4.28 (m, 2H, H₃' and H₄'), 4.14 (s, 3H, 6OCH₃), 4.10-4.05 (m, 1H, CHCH₃), 3.74, 3.72, 3.63, 3.60 (2AB, *J*_{AB}=10.5 Hz, 2H, OCH₂C(CH₃)₃), 1.36, 1.33 (2d, *J*=7.0 Hz, 3H, CHCH₃), 0.96, 0.94 (2s, 3H, 2'CCH₃), 0.86 (s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.01 (d, ³*J*_{C-C-N-P}= 5.0 Hz, C=O), 174.76 (d, ³*J*_{C-C-N-P}= 6.3 Hz, C=O), 162.62 (C6), 154.33 (C2), 153.59, 153.55 (C4), 147.96, 147.90 (ipso Naph), 143.79, 143.50 (C8), 136.22, 136.19 (C10-Naph), 128.84, 128.76 (CH Naph), 127.79, 127.73 (C9-Naph), 127.44, 127.37, 126.46, 125.88, 125.85, 122.71, 122.71 (CH Naph), 121.56 (C5), 116.12, 116.07 (2d, ³*J*_{C-C-O-P}= 3.8 Hz, C2-Naph), 94.02, 93.91 (C1'), 82.70 (d, ³*J*_{C-C-O-P}= 7.5 Hz, C4'), 82.60 (d, ³*J*_{C-C-O-P}= 8.8 Hz, C4'), 80.01, 79.95 (C2'), 75.37 (OCH₂C(CH₃)₃), 74.81, 74.62 (C-3'), 67.97, 67.62 (2d, ²*J*_{C-O-P}= 5.0 Hz, C5'), 59.54 (6OCH₃), 51.81, 51.71 (CHCH₃), 32.25 (OCH₂C(CH₃)₃), 26.72 (OCH₂C(CH₃)₃), 20.91, 20.68 (2d, ³*J*_{C-C-N-P}= 6.3 Hz, CHCH₃), 20.27, 20.24 (2'CCH₃).

HPLC (System 1) *t*_R = 17.65, 17.89 min.

MS (ES+) *m/z*: 677.20 (MH⁺, 100%).

Synthesis of 2-*O*-methyl-6-*O*-methyl-9-(2'-*C*-methyl-β-*D*-ribofuranosyl) purine (10.28).



To a suspension of 2-*C*-chloro-6-*O*-methyl-9-(2'-*C*-methyl-2,3,5-tri-*O*-benzoyl-β-*D*-ribofuranosyl)purine (**10.11**, 1.40 g, 3.06 mmol) in anhydrous methanol (36 ml) at 0 °C NaOMe in methanol (0.99 g, 18.38 mmol) was added. The mixture was stirred at room temperature for 3 days then quenched by addition of

amberlite (H^+). The mixture was then filtrated and solvent was removed under reduced pressure. The residue was purified by silica gel chromatography ($CHCl_3/MeOH$, 85:15) to give the pure compound **10.28** (0.86g, 86 %) as a white solid.

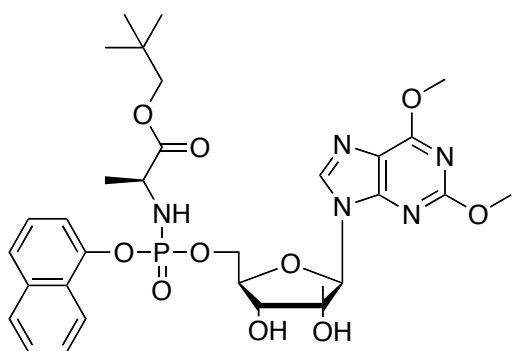
1H (500 MHz, DMSO) δ 8.05 (s, 1H, H_8), 5.93 (s, 1H, $H_{1'}$), 5.25 (s, 1H, 2'OH), 5.23 (bs, 1H, 3'OH), 5.14 (t, $J=5.0$ Hz, 1H, 5'OH), 4.11-4.08 (m, 1H, $H_{3'}$), 4.06 (s, 3H, 6OCH₃), 3.95 (s, 3H, 2OCH₃), 3.94-3.91 (m, 1H, $H_{4'}$), 3.86-3.82 (m, 1H, $H_{5'a}$), 3.72-3.68 (m, 1H, $H_{5'b}$), 0.82 (s, 3H, 2'CCH₃).

^{13}C (126 MHz, DMSO) δ 161.28 (C6), 160.99 (C2), 152.84 (C4), 140.17 (C8), 116.57 (C5), 90.92 (C1'), 82.73 (C4'), 78.49 (C2'), 71.80 (C3'), 59.66 (C5'), 54.72 (2OCH₃), 53.94 (6OCH₃), 19.83 (2'CCH₃).

HPLC (System 1) t_R = 9.36 min

HRMS calculated for $C_{13}H_{18}N_4O_6$: 326.1258; found 326.1247.

Synthesis of 2-C-chloro-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine 5'-O-[α -naphthyl-(2,2-dimethylprooxy-L-alaninyl)] phosphate (10.29a).



Prepared according to the Standard Procedure 7 from: 2-C-chloro-6-O-methyl-9-(2'-C-methyl- β -D-ribofuranosyl) purine (**10.12**, 200 mg, 0.62 mmol), α -naphthyl-(2,2-dimethylprooxy-L-alaninyl) phosphorochloridate (**3.3e**, 406 mg, 1.23 mmol), and *t*BuMgCl (1.23 ml, 1.23

mmol) in 5 ml of dry THF. The crude mixture was purified by column chromatography, using $CHCl_3/MeOH$ (0 to 5%, gradient) as an eluent. To give a pure product **10.25a** as white foam (72 mg, 17%).

^{31}P NMR (202 MHz, MeOD) δ 4.12, 4.08.

1H NMR (500 MHz, MeOD) δ 8.20, 8.19 (2s, 1H, H_8), 8.16, 8.10 (2d, $J=8.0$ Hz, 1H, H_8 -Naph), 7.87, 7.84 (2d, $J=8.0$ Hz, 1H, $H_{5'}$ -Naph), 7.68, 7.65 (2d, $J=7.5$ Hz, 1H, $H_{4'}$ -Naph), 7.54-7.42 (m, 3H, H_7 , H_6 , H_2 -Naph), 7.37, 7.36 (2t, $J=8.0$ Hz, 1H, $H_{3'}$ -Naph),

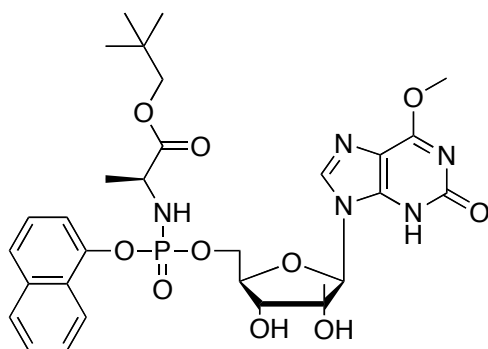
6.06, 6.05 (2s, 1H, H_{1'}), 4.64-4.54 (m, 2H, H_{5'}), 4.41, 4.36 (2d, $J=8.50$ Hz, 1H, H_{3'}), 4.31-4.26 (m, 1H, H_{4'}), 4.12, 4.11 (2s, 3H, 6OCH₃), 4.08-4.04 (m, 1H, CHCH₃), 4.03, 3.97 (2s, 3H, 2OCH₃), 3.75, 3.71, 3.64, 3.63 (2AB, $J_{AB}=10.5$ Hz, 2H, OCH₂C(CH₃)₃), 1.33, 1.32 (2d, $J=7.5$ Hz, 3H, CHCH₃), 1.00, 0.98 (2s, 3H, 2'CCH₃), 0.87, 0.86 (2s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 175.09, 175.06 (C=O), 163.35, 163.26 (C6), 162.99, 162.92 (C2), 154.00, 153.89 (C4), 147.91 (ipso Naph), 141.90, 141.63 (C8), 136.26, 136.22 (C10-Naph), 128.84, 128.76 (CH Naph), 127.73, 127.67 (C9-Naph), 127.43, 127.38, 126.43, 125.88, 122.72, 122.68 (CH Naph), 118.27 (C5), 116.12, 116.09 (2d, $^3J_{C-C-O-P}=3.8$ Hz, C2-Naph), 93.85, 93.72 (C1'), 82.39 (d, $^3J_{C-C-O-P}=7.5$ Hz, C4'), 82.28 (d, $^3J_{C-C-O-P}=8.8$ Hz, C4'), 79.94, 79.87 (C2'), 75.34, 75.31 (OCH₂C(CH₃)₃), 74.87, 74.63 (C3'), 68.12, 67.74 (2d, $^2J_{C-O-P}=5.0$ Hz, C5'), 55.93, 55.85 (2OCH₃), 54.96 (6OCH₃), 51.77, 51.64 (CHCH₃), 32.25 (OCH₂C(CH₃)₃), 26.70, 26.67 (OCH₂C(CH₃)₃), 20.80, 20.60 (2d, $^3J_{C-C-N-P}=6.3$ Hz, CHCH₃), 20.25 (2'CCH₃).

HPLC (System 1) t_R = 19.71, 20.05 min

HRMS calculated for C₃₁H₄₀N₅O₁₀PNa: 696.2484; found 696.2477

Synthesis of 6-*O*-methyl-2'-*C*-methylxanthosine 5'-*O*-[α -naphthyl-(2,2-dimethylpropoxy-L-alaninyl)] phosphate (10.30a).



Prepared according to the Standard Procedure 14, from: **10.29a** (30 mg, 0.045 mmol) was dissolved in dry acetonitrile (5 ml) under argon atmosphere and NaI (14 mg, 0.098 mmol) was added, followed by the addition of trimethylsilyl chloride (0.012 ml, 0.098 mmol).

The reaction mixture was stirred at ambient temperature for 4 h. After work up, the residue was purified by preparative TLC, using CHCl₃/MeOH (96:4) as an eluent. The pure compound **10.30a** was obtained as off white solid (17 mg, 58%).

³¹P NMR (202 MHz, MeOD) δ 4.22.

¹H NMR (500 MHz, MeOD) δ 8.15 (d, $J=8.5$ Hz, H₈-Naph), 8.02 (s, 1H, H₈), 7.85 (d, $J=8.0$ Hz, 1H, H₅-Naph), 7.68 (d, $J=8.0$ Hz, 1H, H₄-Naph), 7.53-7.46 (m, 3H, H₇, H₆,

H₂-Naph), 7.38 (t, $J=8.0$ Hz, 1H, H₃-Naph), 5.99 (s, 1H, H_{1'}), 4.63-4.52 (m, 2H, H_{5'}), 4.29-4.24 (m, 2H, H_{3'}, H_{4'}), 4.05 (q, $J=7.0$ Hz, 1H, CHCH₃), 4.00 (s, 3H, 2OCH₃), 3.78, 3.76, 3.70, 3.65 (AB, $J_{AB}=10.5$ Hz, 2H, OCH₂C(CH₃)₃), 1.34 (d, $J=7.0$ Hz, 3H, CHCH₃), 1.00 (s, 3H, 2'CCH₃), 0.89 (s, 9H, OCH₂C(CH₃)₃).

¹³C NMR (126 MHz, MeOD) δ 173.63 (C=O), 162.56 (C6), 162.99, 162.92 (C2), 154.15, 153.90 (C4), 147.91 (ipso Naph), 139.14 (C8), 136.26 (C10-Naph), 128.81, 128.49 (CH Naph), 127.75 (C9-Naph), 127.48, 126.44, 125.95, 122.78 (CH Naph), 118.37 (C5), 116.16 (d, $^3J_{C-C-O-P}=3.8$ Hz, C2-Naph), 93.61 (C1'), 82.10 (d, $^3J_{C-C-O-P}=8.8$ Hz, C4'), 79.95 (C2'), 75.38 (OCH₂C(CH₃)₃), 74.47 (C3'), 67.60 (d, $^2J_{C-O-P}=5.0$ Hz, C5'), 56.33 (6OCH₃), 51.78 (CHCH₃), 32.28 (OCH₂C(CH₃)₃), 26.72 (OCH₂C(CH₃)₃), 20.59 (d, $^3J_{C-C-N-P}=7.5$ Hz, CHCH₃), 20.33 (2'CCH₃).

HPLC (System 1) t_R = 16.79, 17.01 min.

HRMS calculated for C₃₀H₃₈N₅O₁₀P: 659.2451; found 659.2438.

APPENDIX I

1. McGuigan C, Perrone P, Madela K, Neyts J. The phosphoramidate ProTide approach greatly enhances the activity of β -2'-C-methylguanosine against hepatitis C virus. *Bioorg Med Chem Lett* **2009**, 19 (15), 4316-4320.
2. McGuigan C, Gilles A, Madela K, Aljarah M, Holl S, Jones S, Vernachio J, Hutchins J, Ames B, Bryant KD, Gorovits E, Ganguly B, Hunley D, Hall A, Kolykhalov A, Liu Y, Muhammad J, Raja N, Walters R, Wang J, Chamberlain S, Henson G. Phosphoramidate ProTides of 2'-C-methylguanosine as highly potent inhibitors of hepatitis C virus. Study of their in vitro and in vivo properties. *J Med Chem* **2010**, 53 (13), 4949-4957.
3. McGuigan C, Madela K, Aljarah M, Gilles A, Brancale A, Zonta N, Chamberlain S, Vernachio J, Hutchins J, Hall A, Ames B, Gorovits E, Ganguly B, Kolykhalov A, Wang J, Muhammad J, Patti JM, Henson G. Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus. *Bioorg Med Chem Lett* **2010**, 20 (16), 4850-4854.
4. Vernachio J, Bleiman B, Bryant KD, Chamberlain S, Hunley D, Hutchins J, Ames B, Gorovits E, Ganguly B, Hall A, Kolykhalov A, Liu Y, Muhammad J, Raja N, Walters CR, Wang J, Williams K, Patti JM, Henson G, Madela K, Aljarah M. INX-08189, a phosphoramidate prodrug of 6-O-methyl-2'-C-methyl guanosine, is a potent inhibitor of hepatitis C virus replication with excellent pharmacokinetic and pharmacodynamic properties *Antimicrobial Agents and Chemother* **2011**, 55 (5), 1843-1851.
5. McGuigan C, Madela K, Aljarah M, Gilles A, Battina SK, Ramamurty CVS, Rao CS, Vernachio J, Hutchins J, Hall A, Kolykhalov A, Henson G, Chamberlain S. Dual pro-drugs of 2'-C-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus. *Bioorg Med Chem Lett* **2011**, 21 (19), 6007-6012.
6. McGuigan C, Madela K, Aljarah M, Bourdin C, Arrica M, Barrett E, Jones S, Kolykhalov A, Bleiman B, Bryant KD, Ganguly B, Gorovits E, Henson G, Hunley D, Hutchins J, Muhammad J, Obikhod A, Patti JM, Walters CR, Wang J, Vernachio J, Ramamurty CVS, Battina SK, Chamberlain S. Phosphorodiamidates as a Promising New Phosphate Prodrug Motif for

Antiviral Drug Discovery: Application to Anti-HCV Agents. *J Med Chem* **2011**, 54 (24), 8632-8645.

7. Madela K, McGuigan C. Progress in the development of anti-HCV nucleoside and nucleotide prodrugs. *Future Med Chem* **2012**, 4(5), 625-650.



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl



The phosphoramidate ProTide approach greatly enhances the activity of β -2'-C-methylguanosine against hepatitis C virus

Christopher McGuigan^{a,*}, Plinio Perrone^a, Karolina Madela^a, Johan Neyts^b

^a Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK

^b Rega Institute for Medical Research Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:

Received 20 April 2009

Revised 18 May 2009

Accepted 20 May 2009

Available online 10 June 2009

Keywords:

HCV
ProTide
Phosphoramidate
Nucleoside
Nucleotide
Antiviral
Pro-drug

ABSTRACT

β -2'-C-Methyl purines (**1**, **2**) are known inhibitors of hepatitis C virus (HCV). We herein report the synthesis, biological and enzymatic evaluation of their 5'-phosphoramidate ProTides. Described herein are seven L-alanine phosphoramidate derivatives with variations to the amino acid ester. The 1-naphthyl phosphoramidate of β -2'-methylguanosine containing the benzyl ester (**20**) was the most active at 0.12 μ M, an 84-fold of increase in activity compared to the parent nucleoside (**2**) with no increase of cytotoxicity. The carboxypeptidase mediated hydrolysis of several ProTides showed a predictive correlation with their activity versus HCV in replicon.

© 2009 Elsevier Ltd. All rights reserved.

The hepatitis C virus (HCV) was identified in 1989 as a member of the family of the Flaviviridae.¹ An estimated 180 million people are chronically infected with HCV and thus at increased risk of developing life threatening liver disease (including cirrhosis and hepatocellular carcinoma). HCV infections are the major reason for liver transplantation in industrialized countries. The current therapy for HCV (pegylated interferon and ribavirin) has limited efficacy and major side-effects.² Several examples of modified nucleosides have already been reported with potential anti-HCV activity.^{3,4} Modified nucleosides need to be phosphorylated to their corresponding 5'-triphosphates by the host cell kinases. In many cases, however, nucleoside analogues are poor substrates for the kinases and the pharmacologically active triphosphate species cannot be considered as possible drug candidates due to their high instability and poor cellular permeation.⁵ In many cases, the limiting step in this process is represented by the conversion to the corresponding 5'-monophosphate. Our group has developed in the past the aryloxy-phosphoramidate ProTide approach which allows the delivery of the monophosphorylated nucleoside analogue into the cell, bypassing the need of the first phosphorylation step.⁵ We have previously reported the successful application of the ProTide approach to different nucleoside analogues^{6,7,10,11} 2'-Methylpurines (adenosine and guanosine) have been shown to

be potent anti-HCV agents.³ β -2'-Methyladenosine (**1**) (Fig. 1) showed EC_{50} = 0.3 μ M against HCV in replicon assay, and its corresponding 5'-triphosphate inhibited HCV RNA polymerase at 1.9 μ M. β -2'-Methylguanosine (**2**), instead, showed IC_{50} = 0.13 μ M (inhibition of RdRp), EC_{50} = 3.5 μ M against HCV, but most importantly the detected level of its corresponding 5'-triphosphate was rather poor.³ In the case of β -2'-methylguanosine (**2**), the low level of its intracellular 5'-triphosphate may be an indication that this nucleoside is a poor substrate for nucleoside kinases, limiting its antiviral efficacy.

We decided to apply the aryloxy-phosphoramidate approach to these two nucleoside analogues in order to explore the possibility of further increasing their activity against HCV. In the first instance, β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**) were synthesized and evaluated for their effect on HCV (sub genomic) replicon replication.

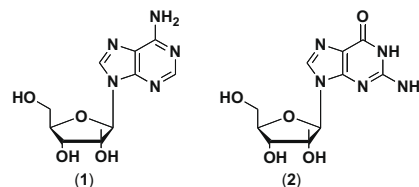


Figure 1. β -2'-Methyladenosine (**1**) and β -2'-methylguanosine (**2**).

* Corresponding author. Tel./fax: +44 0 2920874537.

E-mail address: mcguigan@cardiff.ac.uk (C. McGuigan).

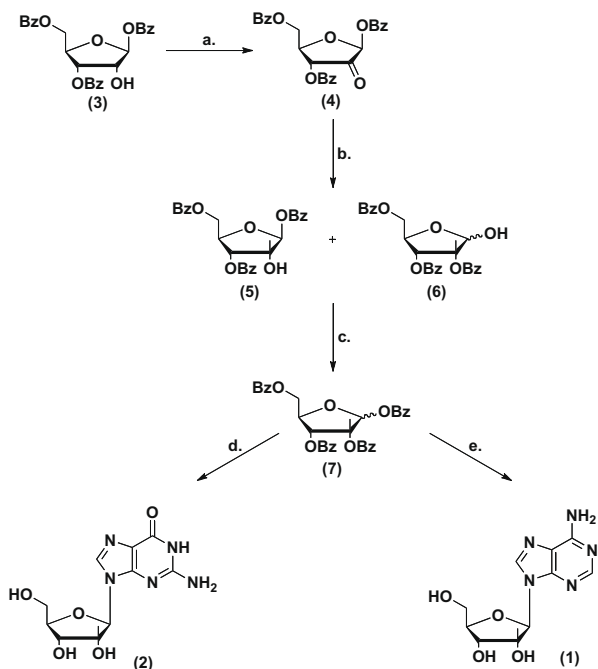


Figure 2. Synthesis of β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**). Reagents: (a) Dess–Martin reagent, CH_2Cl_2 dry; (b) CH_3TiCl_3 , Et_2O dry; (c) BzCl , DMAP, Et_3N ; (d) (1) N2-acethylguanine, HMDS, TMS-triflate, *p*-xylene; (2) $\text{CH}_3\text{OH}/\text{NH}_3$; (e) (1) N6-pivaloyladenine, DBU, TMS-triflate, CH_3CN ; (2) $\text{CH}_3\text{OH}/\text{NH}_3$.

The synthesis of β -2'-methyladenosine (**1**) and β -2'-methylguanosine (**2**) was planned following the reported procedure (Fig. 2).³

The oxidation in the 2-position of the tri-benzoylated ribose (**3**) was performed in the presence of the synthesized Dess–Martin reagent.⁷ The stereoselective addition of the methyl group in the β -2-position was performed by the addition of **4** to a solution of methyl titanium trichloride, synthesized in situ from an anhydrous solution of titanium tetrachloride and methylmagnesium bromide in diethyl ether, to give a mixture of **5** and **6** (Fig. 2).⁸ The next benzoylation reaction was performed under standard conditions to give **7**.³ In the case of β -2'-methyladenosine (**1**), in order to avoid a problem of selectivity in N^7 - and N^9 -positions, it was necessary to protect the NH_2 with the pivaloyl group. The following coupling reaction was performed in the presence of DBU and trimethylsilyl trifluoromethanesulfonate (Fig. 2). Heteronuclear multiple bond correlation (HMBC) showed the correlation between C4 and H1', confirming the presence of the N^9 -regioisomer. Nuclear Overhauser Enhancement Spectroscopy (NOESY), showed no correlation between the proton of H1' and the three protons of the methyl group in the 2'-position confirming the presence of the β -nucleoside; instead, the correlation between H2' and the three protons of the methyl group in the 2'-position confirmed the presence of the methyl group in the β -position. The final step was the removal of the pivaloyl group using $\text{NH}_3/\text{CH}_3\text{OH}$ at room temperature in a sealed tube (Fig. 2). The fully benzoylated β -2'-methyl sugar (**7**) was also the substrate for the synthesis of 2'-methylguanosine (**2**). In order to avoid N^9 - and N^7 -regioisomers, the coupling reaction was performed in two steps: synthesis of the totally silylated acetylguanine and coupling reaction in the presence of the appropriate 2-methylribose sugar (**7**) and trimethylsilyl trifluoromethanesulfonate (TMS-triflate) using *para*-xylene as solvent (Fig. 2).⁹ The N^9 -regioisomer was isolated without any traces of N^7 -isomer. Also in this case the final step required the use of $\text{NH}_3/\text{CH}_3\text{OH}$ to give the desired product (**2**) in quantitative yield (Fig. 2). In our previous work,^{10,11} the phosphoramidate synthesis was greatly improved with the presence of a protecting group in the 2'- and

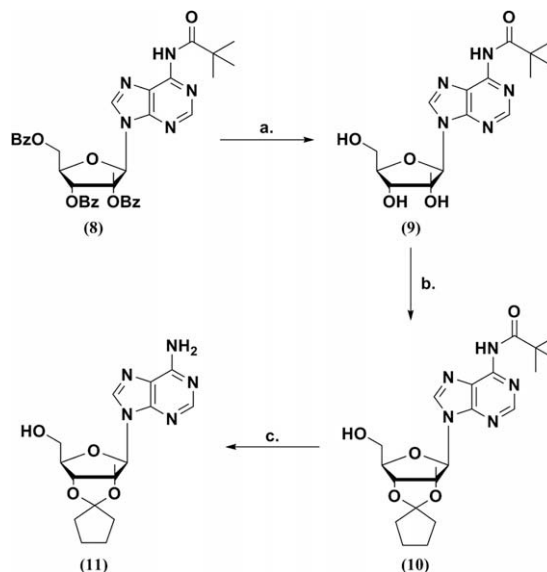


Figure 3. Synthesis of 2',3'-protected- β -2'-methyladenosine (**11**). Reagents: (a) 1 M NaOH, ethanol, pyridine; (b) 1,1-dimethoxypentane, *p*-TSA; (c) NH_3 , CH_3OH .

3'-position of the nucleoside. Consequently, the N^6 -pivaloyl-2',3',5'-tribenzoate- β -2'-methyladenosine (**8**) was selectively deprotected in the 2'-, 3'-, and 5'-position whilst keeping the pivaloyl group in the N^6 -position, followed by introduction of the cyclopentylidene group in the 2'- and 3'-positions in the presence of 1,1-dimethoxypentane and *p*-TSA. The final deprotection of the 6-amino group was performed in the presence of $\text{NH}_3/\text{CH}_3\text{OH}$ at room temperature in a sealed tube (Fig. 3).

The introduction of the cyclopentylidene group was attempted also in the case of β -2'-methylguanosine: this attempt was unsuccessful and consequently the use of isopropylidene as protecting group was considered. The synthesis was performed in the presence of a catalytic amount of perchloric acid in a solution of dry acetone (Fig. 4).

The synthesis of the phosphoramidate was performed following the Uchiyama procedure,¹² in the presence of *t*-butyl magnesium chloride (Figs. 5 and 6).¹³

The sugar deprotection in the case of the cyclopentylidene group (synthesis of β -2'-methyladenosine phosphoramidates, **15–17**) required an acidic hydrolysis using 80% formic acid at room temperature (Fig. 5).¹⁴

The hydrolysis of isopropylidene group (synthesis of β -2'-methyladenosine phosphoramidates, **20–23**), instead, was performed using acetic acid at 90 °C (Fig. 6).¹⁵ The yields of the coupling reaction for the synthesis of β -2'-methyladenosine phosphoramidates (**15–17**) were 40–50%, whilst the deprotection reaction yields were 50–60%. Instead, in the case of the synthesis of β -2'-methylguanosine phosphoramidates (**20–23**) the yields for the coupling reaction ranged from 20–40%, while the yields for the deprotection in

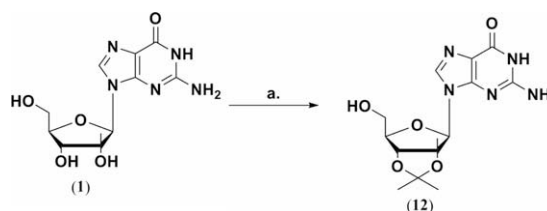


Figure 4. Synthesis of 2',3'-protected- β -2'-methylguanosine (**12**). Reagents: HClO_4 , acetone.

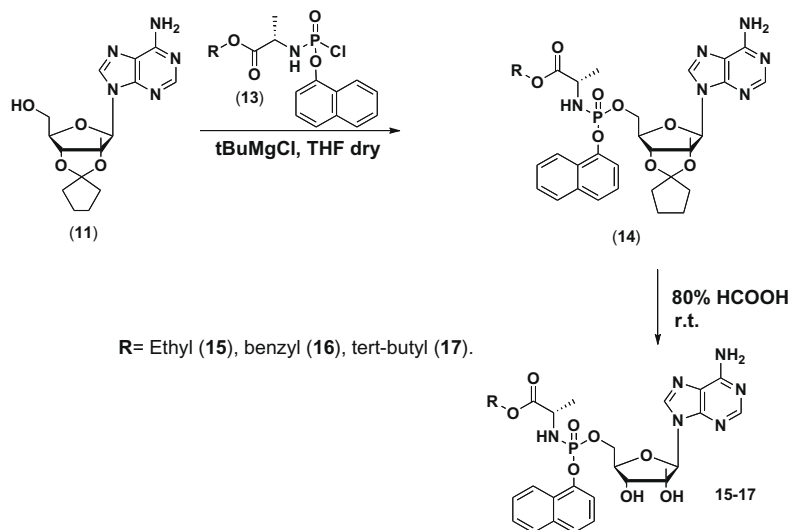


Figure 5. Synthesis of β -2'-methyladenosine phosphoramidates (15–17).

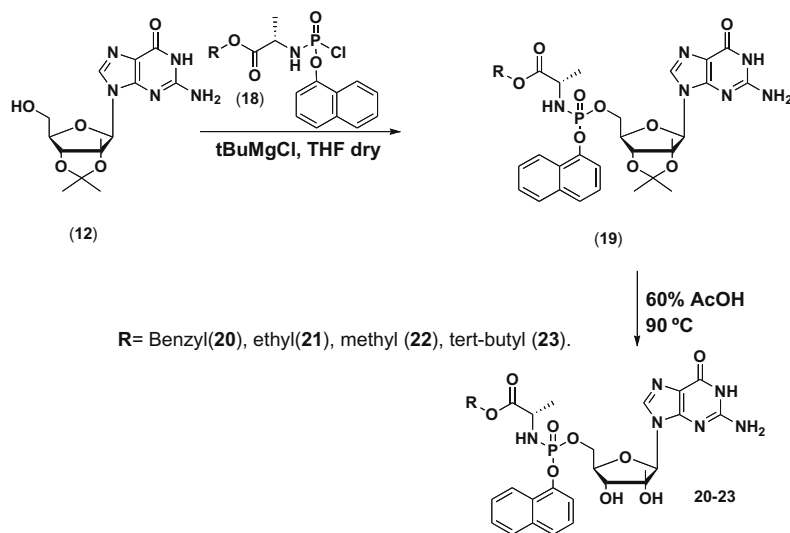


Figure 6. Synthesis of β -2'-methyladenosine phosphoramidates (20–23).

2'- and 3'-positions were 30–50%. In most of the cases, for both nucleosides, purification by column chromatography and a semi-preparative HPLC were required to obtain the pure products. In each case, the phosphoramidates were isolated as mixtures of phosphorus diastereoisomers (ratio 1:1) with two signals present in the ^{31}P NMR spectra.

Each of the phosphoramidates (15–17 and 20–23) and their parent nucleosides (1 and 2) were tested in vitro as inhibitors of HCV replication in the HCV replicon assay using similar conditions as described (Table 1).¹⁶

None of the compounds showed toxicity at 50 μM concentration. The two nucleosides (1 and 2) inhibited HCV replication with EC_{50} values of at 0.25 and 10.1 μM , respectively. The phosphoramidate approach in the case of 1 was unsuccessful, with at best a maintenance of biological activity compared to the parent nucleoside. This result confirms that the conversion of β -2'-methyladenosine (1) to its corresponding 5'-monophosphate in the cell is efficient; consequently, the ProTide approach does not improve this process. However, compound 15 and 16 showed a biological activity similar to their parent nucleoside (1), indicating that in this case, the phosphoramidate approach does successfully deliver the

Table 1

Biological activity of β -2'-methyladenosine (1) and β -2'-methylguanosine (2) and their corresponding phosphoramidates

| Compd | Nucleoside | R | HCV HUH 5-2 | |
|-------|------------|-----------------|------------------------------------|------------------------------------|
| | | | EC_{50} (μM) | CC_{50} (μM) |
| 1 | 2'-Me-A | — | 0.25 | >50 |
| 15 | 2'-Me-A | Ethyl | 0.24 | >50 |
| 16 | 2'-Me-A | Benzyl | 0.27 | >50 |
| 17 | 2'-Me-A | <i>t</i> -Butyl | 4.18 | >50 |
| 2 | 2'-Me-G | — | 10.1 | >50 |
| 20 | 2'-Me-G | Benzyl | 0.12 | >50 |
| 21 | 2'-Me-G | Ethyl | 0.28 | >50 |
| 22 | 2'-Me-G | Methyl | 0.23 | >50 |
| 23 | 2'-Me-G | <i>t</i> -Butyl | 27 | >50 |

5'-monophosphate with a similar efficiency to the nucleoside itself. In contrast, the phosphoramidates of β -2'-methylguanosine (20–22) showed a significant increase in inhibition of HCV replication compared to the parent nucleoside (2). This result supports the notion that: the phosphoramidate approach allows the efficient delivery of the monophosphate of 2 into the replicon cell, 2 is a poor

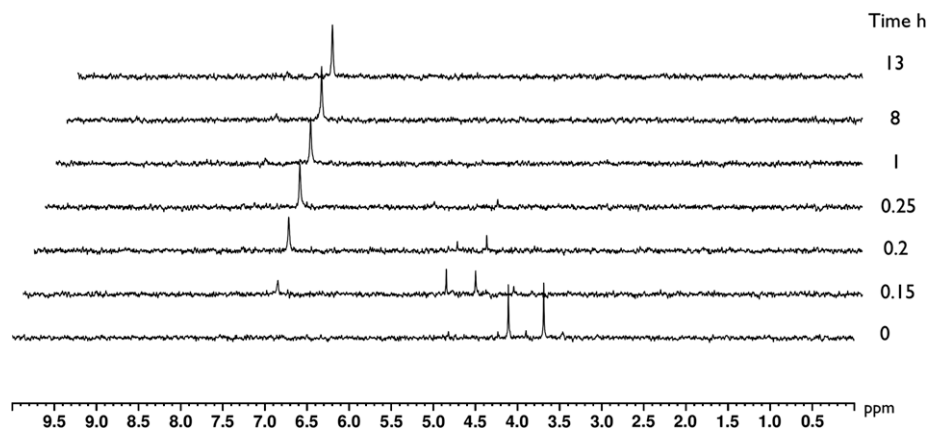


Figure 7. Sequential P-31 NMR scans of (20) incubated with carboxypeptidase Y.¹⁷

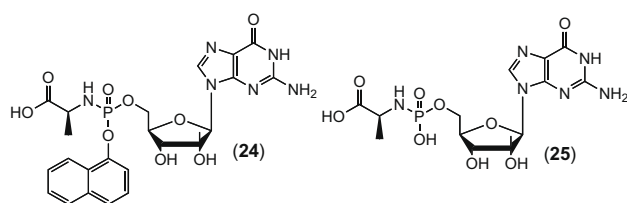


Figure 8. Putative metabolites in the processing of ProTide (20).

substrate for the nucleoside kinases in replicon cells, and the corresponding 5'-monophosphate is efficiently phosphorylated to the corresponding 5'-triphosphate in vitro. Among the four esters, the *t*-butyl (17 and 23) phosphoramidates were found to be less active compared to benzyl, methyl and ethyl esters. This reduced antiviral activity may be related to the relative stability of tertiary esters to enzyme-mediated hydrolysis. The effect is particularly notable for the guanosine series, where the tertiary ester causes a >100-fold loss of activity.

There was no significant difference in antiviral potency considering ethyl, methyl and benzyl esters, indicating that these esters are well tolerated by the enzyme involved in the phosphoramidate conversion. To further understand the high activity of these compounds we carried out an enzyme incubation designed to mimic the first stages of the putative activation of these ProTides in vivo. Thus compound (20) was incubated with carboxypeptidase

Y in TRIZMA buffer and the metabolism of (20) monitored by P-31 NMR.¹⁷ Spectra were recorded at periodic intervals and selected scans are reproduced in Figure 7.

Two P-31 NMR signals are noted for (20) at δ_p ca. 4.0, corresponding to the two phosphate diastereoisomers of the ProTide. After ca. 0.2 h the signals for the parent have disappeared and an intermediate is noted at δ_p ca. 4.5. This material must likely be chiral at the phosphate, as evidenced by the presence of 2 peaks. Parallel work in our laboratories on other compounds indicates this species to be the ester hydrolysed analogue of (20) with a free carboxylate, compound (24).¹⁸ This material is then converted to the final product in this assay, with a single peak at δ_p ca. 7. This is achiral at the phosphate and considered to be the key aminoacyl intermediate, which lacks the ester and aryl moieties; it is the 5'-alaninyl phosphate of (2), compound (25) (Fig. 8).

Our previous work has indicated that this may be a substrate for the HINT family of enzymes, to liberate to free 5'-monophosphate in vivo.¹⁹ As such, its appearance in the enzyme assay is seen as a necessary pre-requisite for antiviral action in vivo. Thus, for comparison purposes we carried out the same study with the poorly active *t*-butyl ester (23). Similar data for the carboxypeptidase assay on (23) are shown in Figure 9.

Several clear differences are noted between the enzyme stability assays of (20) and (23). Most striking is the difference in rate. Thus, while both proceed to give the same putative amino acyl metabolite (25), the *t*-butyl ester (23) is massively slower. The benzyl ester (20) shows only ca. 7% of parent ProTide remaining after 10 min, with a calculated first order half life of 3 min. By

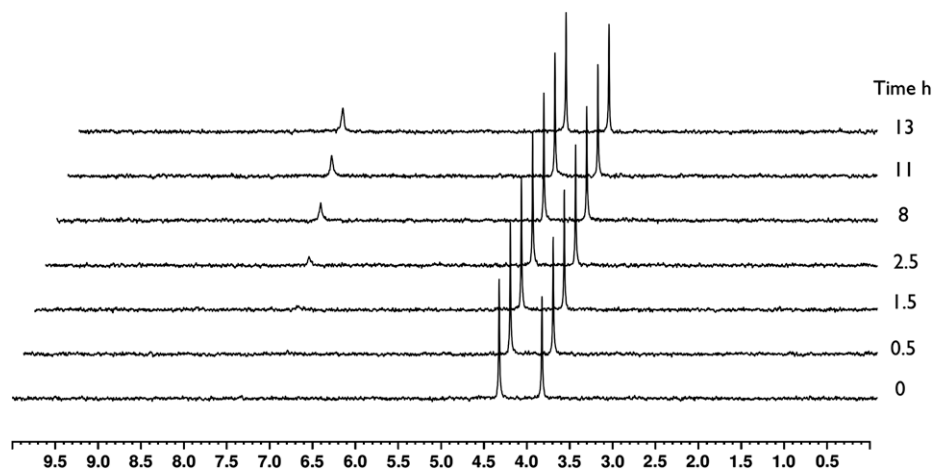


Figure 9. Sequential P-31 NMR scans of (23) incubated with carboxypeptidase Y.

contrast the *t*-butyl ester is only ca. 24% hydrolysed after 13 h, with an estimated half life of 33 h. Thus the (active) benzyl ester is processed ca. 700-fold more efficiently to the key amino acyl metabolite in this assay than is the (poorly active) *t*-butyl ester. Secondly, it is notable that the intermediate (**24**) observed in Figure 7, corresponding to the ester cleaved ProTide, is not observed in Figure 9; presumably the ester cleavage is greatly rate limiting in the case of (**23**) and aryl loss is much more rapid, so intermediate (**24**) never builds up to detectable levels.

In conclusion, we have reported the synthesis of **1** and **2** and a small series of their corresponding phosphoramidates. The most potent compounds were the 1-naphthyl derivatives with benzyl (**20**), ethyl (**21**) or methyl (**22**) ester moieties with 84-fold increase in activity against HCV compared to the parent nucleoside (**2**). The application of phosphoramidate approach to **1** did not significantly improve the activity against HCV compared to the parent nucleoside (**1**). We also note the valuable predictive power of a P-31 NMR based enzyme metabolic assay for the activity of these ProTides versus HCV in vitro.

Acknowledgments

We would like to thank Helen Murphy for secretarial assistance and Katrien Geerts for excellent technical assistance. This work is supported by a grant of the Flemish Fund of Scientific Research (FWO) and VIRGIL, the European Network of Excellence on Antiviral Drug Resistance (Grant LSHM-CT-2004-503359 from the Priority 1 'Life Sciences, Genomics and Biotechnology').

Supplementary data

Supplementary data (additional spectroscopic and analytical data on the target compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.122.

References and notes

- Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W.; Houghton, M. *Science* **1989**, *244*, 359.
- Huang, Z.; Murray, M. G.; Secrist, J. A., III *Antiviral Res.* **2006**, *71*, 351.
- (a) Eldrup, A. B.; Allerson, C. R.; Bennett, C. F.; Bera, S.; Bhat, B.; Bhat, N.; Bosserman, M. R.; Brooks, J.; Burlein, C.; Carroll, S. S.; Cook, D. P.; Getty, K. L.; MacCross, M.; McMasters, D. R.; Olsen, D. B.; Prakash, T. P.; Prhave, M.; Song, Q.; Tomassini, J. E.; Xia, J. *J. Med. Chem.* **2004**, *47*, 2283; (b) Eldrup, A. B.; Prhave, M.; Brooks, J.; Bhat, B.; Prakash, T. P.; Song, Q.; Bera, S.; Bhat, N.; Dande, P.; Cook, D. P.; Bennett, C. F.; Carroll, S. S.; Ball, R. G.; Bosserman, M.; Burlein, C.; Colwell, L. F.; Fay, J. F.; Flores, O. A.; Getty, K. L.; LaFemina, R. L.; Leone, J.; MacCross, M.; McMasters, D. R.; Tomassini, J. E.; Von Langen, D.; Wolanski, B.; Olsen, D. B. *J. Med. Chem.* **2004**, *47*, 5284.
- Clark, J. L.; Hollecker, L.; Mason, J. C.; Stuyver, L. J.; Tharnish, P. M.; Lostia, S.; McBrayer, T. R.; Schinazi, R. F.; Watanabe, K. A.; Otto, M. J.; Furman, P. A.; Stec, W. J.; Patterson, S. E.; Pankiewicz, K. W. *J. Med. Chem.* **2005**, *48*, 5504.
- Hersh, M. R.; Kuhn, J. G.; Phillips, J. L. *Cancer Chemother. Pharmacol.* **2004**, *4*, 371.
- Cahard, D.; McGuigan, C.; Balzarini, J. *Mini-Rev. Med. Chem.* **2004**, *4*, 371.
- (a) McGuigan, C.; Cahard, D.; Sheeka, H. M.; Le Clercq, E.; Balzarini, J. *J. Med. Chem.* **1996**, *39*, 1748; (b) McGuigan, C.; Sheeka, H. M.; Mahmood, N.; Hay, A. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1203; (c) McGuigan, C.; Wedgwood, O. M.; De Clercq, E.; Balzarini, J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2359.
- Harry-O'kuru, R. E.; Smith, J. M.; Wolfe, M. S. *J. Org. Chem.* **1997**, *62*, 1754.
- Li, N.-S.; Piccirilli, J. A. *J. Org. Chem.* **2006**, *71*, 4018.
- Perrone, P.; Daverio, F.; Valente, R.; Rajyaguru, S.; Martin, J. A.; L  v  que, V.; Le Pogam, S.; Najera, I.; Klumpp, K.; Smith, D. B.; McGuigan, C. *J. Med. Chem.* **2007**, *50*, 5463.
- Perrone, P.; Luoni, G. M.; Kelleher, M. R.; Daverio, F.; Angell, A.; Mulready, S.; Congiatu, C.; Rajyaguru, S.; Martin, J. A.; L  v  que, V.; Le Pogam, S.; Najera, I.; Klumpp, K.; Smith, D. B.; McGuigan, C. *J. Med. Chem.* **2007**, *50*, 1840.
- Uchiyama, M.; Aso, Y.; Noyori, R.; Hayakawa, Y. *J. Org. Chem.* **1993**, *58*, 373.
- Standard procedure for the synthesis of 2',3'-protected phosphoramidates*: BuMgCl (2.0 mol equiv, 1 M solution in dry THF) and the appropriate nucleoside (**11** or **12**, 1.0 mol equiv) were dissolved in dry THF (31 mol equiv) and stirred for 15 min. Then a 1 M solution of the appropriate phosphorochloridate (2.0 mol equiv, **13** or **19**) in dry THF was added dropwise, then stirred for 14 h. A saturated solution of NH₄Cl was added and the solvent was removed under reduced pressure to give a yellow solid, which was purified by column chromatography using CHCl₃/MeOH (from 95/5) as eluent. The appropriate fractions were collected and the solvent was removed under reduced pressure to give a white solid.
- Standard procedure for the deprotection of β-2'-methyladenosine phosphoramidates*: the appropriate 2',3'-O,O-cyclopentylidene-β-2'-methyladenosine phosphoramidate (1.0 mol equiv) was added to a solution of formic acid (80% v/v solution in water). The reaction was stirred at rt for 6 h. The solvent was removed under reduced pressure and the obtained yellow oil was subsequently purified by column chromatography using CHCl₃/MeOH (95:5) as eluent followed by a semipreparative HPLC to give a white solid.
- Standard procedure for the deprotection of β-2'-methylguanosine phosphoramidates*: the appropriate 2',3'-isopropylidene-β-2'-methylguanosine phosphoramidates (1.0 mol equiv) was added to a solution of 60% v/v of acetic acid in water at 90 °C for 15 h. The solvent was removed under reduced pressure and the obtained yellow oil was subsequently purified by column chromatography using CHCl₃/MeOH (95:5) as eluent followed by a semipreparative HPLC to give a white solid. Key spectroscopic data on (**20**) (NMR assignments confirmed by 2D spectra): δ_p (CH₃OH-d₄): 4.25, 4.14; δ_H (CH₃OH-d₄): 8.17 (1H, m, H8-guanosine), 7.88 (1H, m, CH-naphthyl), 7.79 (1H, m, CH-naphthyl), 7.53 (2H, m, CH-naphthyl, CH-benzyl), 7.42–7.40 (1H, m, CH-naphthyl), 7.36–7.21 (7H, m, CH-naphthyl, CH-benzyl), 6.05 (1H, d, H1'-guanosine, J = 8.4 Hz), 5.15–4.90 (2H, m, CH₂-benzyl), 4.58–4.49 (2H, d, H3'-guanosine, H4'-guanosine), 4.44–4.34 (2H, m, H5'-guanosine), 4.17–4.11 (1H, m, CHα), 1.37 (3H, m, CH₃-alanine), 1.00 (3H, s, CH₃-2'-guanosine). MS (ES) *m/z*: 687.2 (MNa⁺, 100%); accurate mass: C₃₁H₃₃N₆O₉NaP required 687.1954, found 687.1944. Spectroscopic and Analytical data on other analogues are listed in SI.
- Klumpp, K.; Leveque, V.; Le Pogam, S.; Ma, H.; Jiang, W. R.; Kang, H.; Granycome, C.; Singer, M.; Laxton, C.; Hang, J. Q.; Sarma, K.; Smith, D. B.; Heindl, D.; Hobbs, C. J.; Merrett, J. H.; Symons, J.; Cammack, N.; Martin, J. A.; Devos, R.; Najera, I. *J. Biol. Chem.* **2006**, *281*, 3793.
- Carboxypeptidase Y (Sigma CAS No. 9046-67-7, EC 3.4.16.1, 0.5 mg) was suspended in 200 μL Trizma buffer and added to the ProTide (5 mg) in 200 μL of acetone-d₆ and 400 μL of Trizma, and the reaction followed by ³¹P NMR.
- Valente, R. PhD Thesis, Cardiff University, 2009.
- Congiatu, C.; Brancale, A.; McGuigan, C. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 1121.

Phosphoramidate ProTides of 2'-C-Methylguanosine as Highly Potent Inhibitors of Hepatitis C Virus. Study of Their in Vitro and in Vivo Properties

Christopher McGuigan,^{*,†} Arnaud Gilles,[†] Karolina Madela,[†] Mohamed Aljarah,[†] Sabrina Holl,[†] Sarah Jones,[†] John Vernachio,[‡] Jeff Hutchins,[‡] Brenda Ames,[‡] K. Dawn Bryant,[‡] Elena Gorovits,[‡] Babita Ganguly,[‡] Damound Hunley,[‡] Andrea Hall,[‡] Alexander Kolykhalov,[‡] Yule Liu,[‡] Jerry Muhammad,[‡] Nicholas Raja,[‡] Robin Walters,[‡] Jin Wang,[‡] Stanley Chamberlain,[‡] and Geoffrey Henson[‡]

[†]Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, U.K., and

[‡]Inhibitex Inc., 9005 Westside Parkway, Alpharetta, Georgia 30004

Received March 26, 2010

Hepatitis C virus infection constitutes a serious health problem in need of more effective therapies. Nucleoside analogues with improved exposure, efficacy, and selectivity are recognized as likely key components of future HCV therapy. 2'-C-Methylguanosine triphosphate has been known as a potent inhibitor of HCV RNA polymerase for some time, but the parent nucleoside is only moderately active due to poor intracellular phosphorylation. We herein report the application of phosphoramidate ProTide technology to bypass the rate-limiting initial phosphorylation of this nucleoside. Over 30 novel ProTides are reported, with variations in the aryl, ester, and amino acid regions. L-Alanine compounds are recognized as potent and selective inhibitors of HCV in replicon assay but lack rodent plasma stability despite considerable ester variation. Amino acid variation retaining the lead benzyl ester moiety gives an increase in rodent stability but at the cost of potency. Finally L-valine esters with ester variation lead to potent, stable compounds. Pharmacokinetic studies on these agents in the mouse reveal liver exposure to the bioactive triphosphate species following single oral dosing. Systemic exposure of the ProTide and parent nucleoside are low, indicating possible low toxicity in vivo, while liver concentrations of the active species may be predictive of efficacy in the clinic. This represents one of the most thorough cross-species studies of ProTides to date.

Introduction

An estimated 200 million people, or ca. 3.5% of the world's population, are chronically infected with the hepatitis C virus (HCV^a) and at risk of developing life threatening liver disease such as cirrhosis or liver carcinoma. HCV infection is the major cause of liver transplantation in industrialized countries. The current therapy for HCV consists of pegylated interferon and ribavirin, neither of which are specific inhibitors of HCV, and a regimen which has side effects and limited efficacy in at least half of the patient population.¹

The HCV genome offers several clear targets for specific anti-HCV therapy; these include an RNA polymerase and a serine protease, both of which have attracted considerable academic and industrial attention.²

The RNA dependent HCV polymerase is considered to be an attractive target for therapy on account of a high degree of conservation across the six HCV genotypes, and agents targeted at the active site, such as nucleoside analogues, may be particularly advantageous with respect to the barrier to resistance.³

Several families of nucleoside analogues have emerged with apparent selectivity for HCV, these include 4'-modified agents such as 4'-azidocytidine (R1479, **1a**, Figure 1) developed as an oral pro-drug by Roche⁴ and 2'-C-methyl adenosine (**1b**, Figure 1) and related deaza compounds developed by Merck.⁵

Interestingly, the Merck team observed that the guanine analogue **1c** was more potent (> 10-fold) as an inhibitor of the HCV RNA polymerase, as its bioactive 5'-triphosphate form, than was the triphosphate of the adenosine lead **1b**. However, poor cell entry by **1c** coupled with apparently poor phosphorylation lead to it being > 10-fold less active than **1b** in HCV replicon (**1b** EC₅₀ 0.26 μM, **1c** 3.5 μM).⁵

Unfortunately, despite the reasonable in vitro potency and selectivity of **1b**, the compound could not be progressed due to it being a good substrate for metabolic enzymes (adenosine deaminase and purine nucleoside phosphorylase), leading to low oral bioavailability.⁵

To overcome these PK liabilities of **1b**, the Merck team progressed with several unnatural base modifications, such as the 7-deaza family, leading to their late preclinical candidate MK-0608. As recently highlighted, a lack of data on this family for over three years now may place a query over their progression.³ We were concerned that such base modifications could carry with them the possibility of toxicity, and we sought to retain a natural base and to seek to overcome the limitations of the guanosine compound **1c** in particular. Because the first phosphorylation of **1c** was considered to be rate-limiting, we

*To whom correspondence should be addressed. Phone/Fax: +44 29 20874537. E-mail: mcguigan@cardiff.ac.uk.

^a Abbreviations: HCV, hepatitis C virus; TLC, thin layer chromatography; HPLC, high performance, liquid chromatography; ClogP, calculated logarithm of octanol–water partition coefficient; PK, pharmacokinetics; SI, selectivity index; DNAUC, dose normalized area under the curve; NTP, nucleoside triphosphate; THF, tetrahydrofuran.

wondered if a pro-drug of the 5'-monophosphate of **1c** may be active, and thus we applied our phosphoramidate ProTide approach⁶ to this nucleoside. We first introduced this ProTide motif in 1996⁷ and have exemplified it considerably,⁶ including very recently to **1a**⁸ and briefly to **1b–c**.⁹ Our approach has also been adopted by Pharmasset for their 2'-modified nucleoside HCV family¹⁰ and adapted to acyclic nucleoside phosphonates by Gilead.¹¹ The Pharmasset nucleoside is also 2'-C-methyl based, but is a uridine compound, also with a 2'- α -fluorine. This company are using a ProTide based on isopropylalanine phenyl phosphate,¹² rather parallel to our early work on anti-HIV ProTides¹³ Idenix have also pursued a

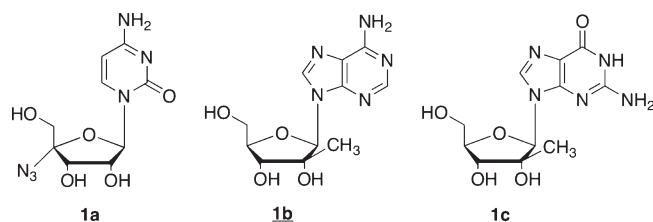


Figure 1. Some anti-HCV nucleosides.

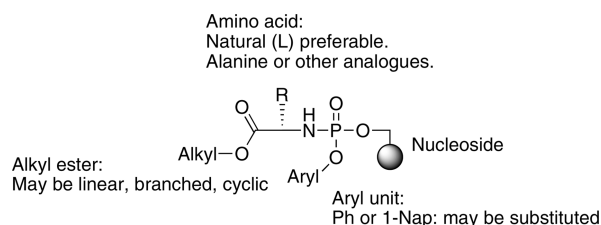
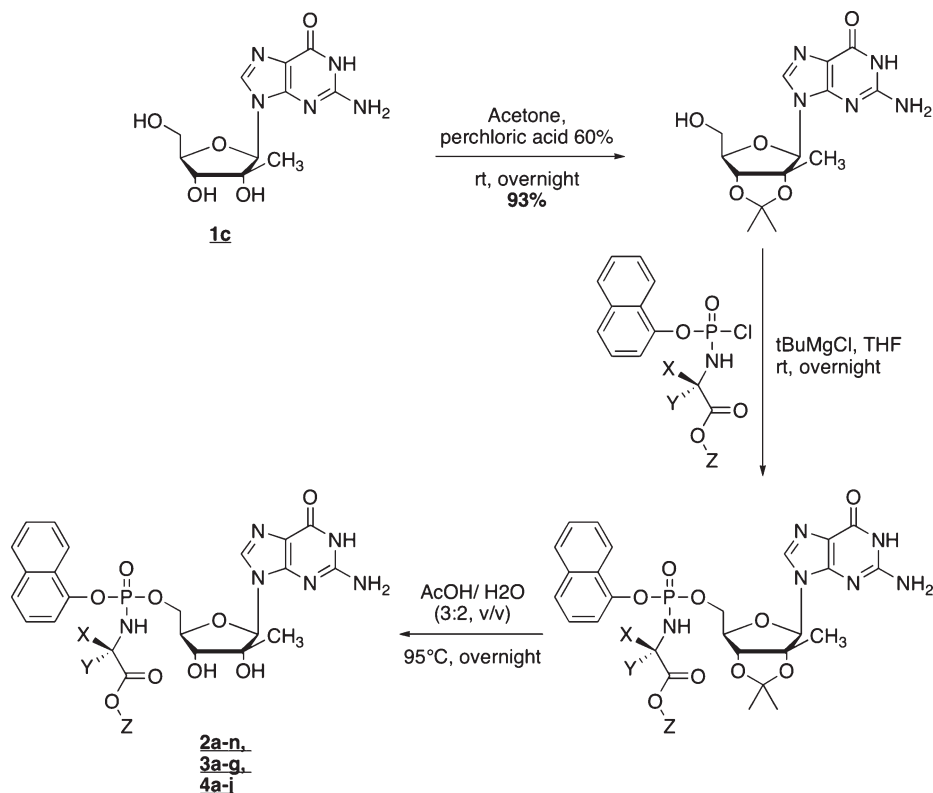


Figure 2. A general ProTide structure.

Scheme 1^a



^a For **2a–n**, **3a–g**, **4a–j**, see Tables 1, 3, 5.

ProTide approach on **1c**, although the precise structure of their lead IDX-184 has yet to issue.¹⁴

Following an early indication that the ProTides may successfully impact on **1c**,⁹ we herein report an extensive study thereof. We report over 30 novel analogues with extensive variation in the aryl, ester, and amino acid regions of the ProTide, and we study these extensively across species for their stability. We note that the benzyl alanine motif continues^{7–9} to exhibit good potency in replicon, but there is poor stability and PK in this series, which can only be solved by a novel combined variation in both the amino acid and ester regions as we will describe. The basic structure of a phosphoramidate motif is shown in Figure 2.

Chemistry

The target ProTides of **1c** were prepared using phosphorochloridate chemistry as we have extensively reported.^{6–9} To aid in both the 5'-regioselectivity of phosphorylation and the general organic solubility of the nucleoside, we investigated various protecting groups for the 2',3'-diol unit. One key issue is the ability to remove this protecting group after construction of the 5'-ProTide unit, and the relative chemical fragility of this group dictates the nature of the diol protecting group that might be acceptable. Indeed, the somewhat surprising acid-stable nature of ProTides¹⁵ means that acid sensitive diol protection may be acceptable, and this led us to the use of isopropylidene protection.

Thus **1c** was allowed to react with acetone and perchloric acid at ambient temperature overnight to give **1d** (Scheme 1) in 93% yield. This proved to be significantly more soluble than **1c** and readily reacted with the appropriate phosphorochloridate reagents^{6–9,15} in organic solvents such as THF (Scheme 1). Various bases could be used to aid the reaction; the Grignard

Table 1. HCV Replicon Activity of L-Alanines Ester Derivatives

| ProTide | aryl | ester | isomers ^a | EC ₅₀ /μM ^b | CC ₅₀ /μM |
|-----------|-------|-----------------------------------|-----------------------------|-----------------------------------|----------------------|
| 1c | | | | 3.5 | > 100 |
| 2a | 1-Nap | Me | 38:62 | 0.21 | > 50 |
| 2b | 1-Nap | nBu | 43:57 | 0.10 | > 100 |
| 2c | 1-Nap | nPnt | 37:63 | 0.091 | ND |
| 2d | 1-Nap | iPr | 40:60 | 0.17 | > 100 |
| 2e | 1-Nap | 2-Bu | 38:62 | 0.12 | > 100 |
| 2f | 1-Nap | cPnt | 48:52 | 0.07 | > 100 |
| 2g | 1-Nap | cHx | 34:66 | 0.045 | > 100 |
| 2h | 1-Nap | tBuCH ₂ | 31:69 | 0.057 | > 100 |
| 2i | 1-Nap | Bn | 45:55 | 0.062 | > 100 |
| 2j | 2-Nap | Bn | 43:57 | 0.17 | > 50 |
| 2k | 1-Nap | BnCH ₂ | 44:56 | 0.09 | > 100 |
| 2l | 1-Nap | BnCH ₂ CH ₂ | 40:60 | 0.17 | > 100 |
| 2m | 1-Nap | R/S PhEt | 33, 14, 35, 18 ^c | 0.095 | > 100 |
| 2n | 1-Nap | S PhEt | 42:58 | 0.08 | > 100 |

^a Isomer ratio from HPLC; polar:nonpolar. ^b Replicon data for genotype 1b in HUH7 cells with 48 h exposure. ^c Four stereoisomers.

reagent *t*BuMgCl was among the most effective, and preferably used in ca. 2 molar equivalence, along with the phosphorochloridate. Reaction overnight at ambient temperature followed by flash column chromatography on silica gave the isopropylidene protected intermediates (Scheme 1) in 30–88% yield. These were deprotected using 60% acetic acid at 95 °C overnight. The relative stability of the ProTide motif under these conditions was taken as further support of their surprising acid stability and may be taken as further evidence of their possible compatibility with oral dosing. The final compounds **2a–n**, **3a–g**, and **4a–j** (Tables 1, 3, and 5) were each isolated after column chromatography, sometimes followed by preparative TLC and/or HPLC. Standard methods are given below and spectroscopic and analytical data on **2a** only; full detail on other analogues is given as Supporting Information. In each case, the final products were isolated as mixtures of diastereoisomers at the phosphate, as evidenced by multiple (in most cases 2) peaks in the P-31 NMR and also in the HPLC. The approximate ratio of peaks (fast eluting on reverse phase, i.e., polar to nonpolar) as determined by HPLC is given in Tables 1, 3, and 5, where peaks were sufficiently clearly resolved. The approximate ratios from P-31 NMR roughly mirrored those determined by HPLC and allowed a correlation between HPLC mobility/polarity and NMR shift, such that the most predominant isomer in most cases was the downfield NMR peak, corresponding to the more nonpolar stereoisomer. The HPLC retention time data confirmed that the ProTides were in every case considerably more lipophilic than parent **1c**. Calculated lipophilicity (ClogP) values¹⁶ for **1c** and **2a** are –2.6 and –0.52, respectively, indicating a roughly 100-fold lipophilicity enhancement for **2a**. Because Merck had noted apparently poor cell uptake by **1c**,⁵ we considered the enhancement in this parameter for the ProTides potentially useful in vivo. However, the figure for the methylalanine ester **2a** was still somewhat lower than that considered optimal for passive cell uptake, and thus we varied the ester moiety to enhance lipophilicity further and also to probe the putative ester cleavage step considered to be key to the in vivo activation of these agents.^{6,15} In the L-alanine series **2a–n**, the lengthening, branching, and aromatization of the ester moiety lead to agents such as the benzyl ester **2i** with a ClogP of 1.2 and branched analogues of this, such as **2m** and **2n**, with perhaps near ideal values of 1.5. Notably, in the case of **2m**, the introduction of a further variable chiral center in the ester led to a mixture of four diastereomers, as evidenced by P-31 NMR and HPLC.

Table 2. Plasma Stability of L-Alanine Esters^a

| ProTide | ester | time | % remaining in plasma at 2–4 °C | | | | |
|-----------------------|--------------------|--------|---------------------------------|------|--------|-----|-------|
| | | | human | cyno | canine | rat | mouse |
| 2a | Me | 30 min | ND | ND | ND | ND | 1.7 |
| 2b | nBu | 30 min | 100 | 98 | 95 | 0.4 | 0 |
| | | 4 h | 97 | 81 | 90 | 0 | 0 |
| 2c | nPnt | 30 min | 100 | 94 | 98 | 0 | 0 |
| | | 4 h | 96 | 94 | 98 | 0 | 0 |
| 2d | iPr | 30 min | ND | ND | ND | ND | 14 |
| 2e | 2-Bu | 30 min | ND | ND | ND | ND | 10 |
| 2g | cHx | 30 min | 100 | 100 | 100 | 2.5 | 2.9 |
| | | 4 h | 100 | 100 | 100 | 0.5 | 0 |
| 2h | tBuCH ₂ | 30 min | ND | ND | ND | ND | 0.3 |
| 2i | Bn | 30 min | 58 | 54 | 67 | 1.3 | 1.1 |
| 2j^b | Bn | 30 min | ND | ND | ND | ND | 0.7 |
| 2m | (R/S)-1-PhEt | 30 min | 86 | 100 | 97 | 0.7 | 2.2 |
| | | 4 h | 86 | 88 | 93 | 0.3 | 0 |
| 2n | S-PhEt | 30 min | 100 | 100 | 97 | 0.8 | 3.1 |
| | | 4 h | 99 | 100 | 92 | 0 | 0 |

^a ND: not determined. ^b 2-Naphthyl.

Besides ester variation, we prepared one analogue, **2j**, with 2-naphthyl as the aryl moiety in place of 1-naphthyl. We also varied the amino acid unit from L-alanine (**2a–n**), to valine (**3a**), leucine (**3b**), methionine (**3c–d**), phenylalanine (**3e**), isoleucine (**3f**), and the unnatural achiral amino acid dimethylglycine (**3g**) (Table 3). In most cases, these were prepared as the benzyl ester, except for **3d**, being the *i*Pr ester of Met.

As will be discussed below, L-valine emerged as a promising amino acid from these earlier studies. This is notable, as it had previously been observed by us to be among the least effective amino acids when applied to several families of ProTides.^{6,17} However, in this case, promising biological data necessitated the preparation of a family of L-valine esters (**4a–j**, Table 5). These included some unusual esters not previously widely reported for ProTides: the substituted benzyl family (**4f–i**) and the neopentyl compound (**4j**).

Activity in Replicon Assay and Plasma Stability

As is common in anti-HCV drug development, we used subgenomic HCV replicon as a primary biological readout. Data are reported for the initial L-alanine family **2a–n** in Table 1, along with comparator data for **1c** in this assay. Each data point represents the mean of at least three independent assays. As noted in Table 1, all of the L-alanine ProTides were active sub-μM in the replicon assay, while **1c** was only active at 3.5 μM. Thus, the initial family of esters was 4–50-fold more potent than **1c**. This was taken as an early indication that the ProTides successfully improved the intracellular delivery of the eventual 5'-triphosphate pharmacophore of **1c**, presumably by a combination of enhanced passive diffusion of the lipophilic ProTides into cells and the delivery of the monophosphate therein by a nucleoside kinase-independent process. There was no clear correlation between ProTide lipophilicity and biological potency in this family, with several diverse esters showing activity in the 40–60 nM range.

All the ProTides displayed minimal toxicity in the Huh7 cells expressing the HCV genotype 1b bicistronic subgenomic replicon. When replicon 1b cells were incubated with the different ProTides for 72 h and cell viability was measured using the CellTiter-Glo assay (Promega, Madison, WI), CC₅₀ values greater than 100 μM were routinely observed. This was true even for the most potent compounds like **2g**, thus leading

Table 3. HCV Replicon Activity of Different Amino Acid Derivatives^a

| ProTide | aryl | ester | AA | isomers ^b | EC ₅₀ /μM ^b | CC ₅₀ /μM |
|-----------|-------|-------|---------------------|----------------------|-----------------------------------|----------------------|
| 3a | 1-Nap | Bn | L-Val | 32:68 | 0.76 | > 100 |
| 3b | 1-Nap | Bn | L-Leu | NA ^c | 0.12 | > 50 |
| 3c | 1-Nap | Me | L-Met | 31:69 | 0.28 | > 100 |
| 3d | 1-Nap | iPr | L-Met | 36:64 | 0.34 | > 100 |
| 3e | 1-Nap | Bn | L-Phe | 34:66 | 1.2 | > 100 |
| 3f | 1-Nap | Bn | L-Ile | NA ^c | 0.9 | > 50 |
| 3g | 1-Nap | Bn | Me ₂ Gly | 31:69 | 1.5 | ND |

^aNA: not available. ND: not determined. ^bSee Table 1. ^cPeaks merged on HPLC.

to high selectivity indexes in these cells. Several compounds were also tested against MT4, CEM, HepG2, Huh7, HEL, 293, IEC-6, and CaCo2 cells. All cell lines tested were in log phase of growth throughout the cell cytotoxicity experiments (data not shown). A similar lack of toxicity was observed in these diverse cell lines. For example, compound **2n** had CC₅₀ values > 100 μM for all the above cell lines except MT4 (CC₅₀ = 87 μM) and CEM (CC₅₀ = 82 μM). This gives **2n** an SI of ca. ≥ 1000.

Thus, to progress hit to lead selection, we sought other assays that may be informative of in vivo PK. Prior to investigating the PK of the various ProTides, we examined the plasma stability of the L-alanine esters in mouse, rat, dog, monkey, and human plasma (Table 2). The plasma stability studies were carried out at 2–4 °C because they were intended to confirm sufficient stability for calibration curves, not necessarily for understanding stability at the physiologically more relevant 37 °C. Perhaps not surprisingly, the various esters of the L-alanine ProTides all were very unstable in rodent plasma even at these lower temperatures. The data are reported as % remaining after either 30 min or 4 h. All the L-alanine esters synthesized were essentially completely cleaved in 30 min. The most stable esters were the branched isopropyl and 2-butyl esters, which still were greater than 80% consumed in 30 min even at this lower temperature. Because of this profound plasma instability, it became clear we would not be able to generate calibration curves in rodent plasma and as a result we would not be able to measure ProTide plasma concentrations in either mouse or rat. On the other hand, the L-alanine esters for the most part had sufficient plasma stability in other species to generate calibration curves and measure exposure. The benzyl ester **2i** was the notable exception, with nearly 40% degradation in 30 min in human, monkey, and dog plasma. However, substitution at the benzylic position, as in the case of the 2-phenylethyl derivative **2m** and **2n**, led to substantially improved plasma stability in these three species with up to 100% remaining even after 4 h.

Because modification of the ester group did not readily provide rodent plasma stability, and because being able to do initial pharmacokinetic studies in rodents was considered desirable, the amino acid core of the ProTides was varied to investigate both potency and plasma stability. Thus, as noted in Table 3, we prepared several ProTides, mainly as their benzyl esters, of six different amino acids. Benzyl esters were selected because we believed they provided the best chance of showing potency in the replicon assay. We particularly selected bulky amino acids, partly to enhance lipophilicity and partly to seek to induce some steric hindrance to degradation of the ProTide. As noted in Table 3, this led to a significant (2–10-fold) loss of activity in the best cases and a 20-fold decrease in the worst case. Disubstitution of the α carbon of the amino acid led to a 20-fold decrease in activity, while

Table 4. Plasma Stabilities of Amino Acid Derivatives

| ProTide | ester | AA | time | % remaining in plasma at 2–4 °C | | | | |
|-----------|-------|---------------------|--------|---------------------------------|------|--------|-----|-------|
| | | | | human | cyno | canine | rat | mouse |
| 3a | Bn | L-Val | 30 min | | | | | 89 |
| | | | 4 h | | | | | 66 |
| 3b | Bn | L-Leu | 30 min | | | | | 2 |
| 3c | Me | L-Met | 30 min | | | | | 34 |
| 3d | iPr | L-Met | 30 min | 77 | 96 | 100 | 2.3 | 76 |
| | | | 4 h | 79 | 93 | 100 | 0.5 | 32 |
| 3e | Bn | L-Phe | 30 min | | | | | 1 |
| 3f | Bn | L-Ile | 30 min | | | | | 100 |
| | | | 4 h | | | | | 95 |
| 3g | Bn | Me ₂ Gly | 30 min | 100 | 92 | 100 | 0 | 0 |
| | | | 4 h | 41 | 83 | 100 | 0 | 0 |

Table 5. HCV Replicon Activity of L-Valine Ester Derivatives^a

| ProTide | aryl | ester | AA | isomers ^b | EC ₅₀ /μM ^b | CC ₅₀ /μM |
|-----------|-------|------------------------------------|-------|----------------------|-----------------------------------|----------------------|
| 3a | 1-Nap | Bn | Val | 32:68 | 0.76 | > 100 |
| 4a | 2-Nap | Bn | Val | 56:44 | 1.7 | > 100 |
| 4b | 1-Nap | Bn | D-Val | 68:32 | > 3 | > 100 |
| 4c | 1-Nap | cHx | Val | 65:35 | 1.0 | > 100 |
| 4d | 1-Nap | (R/S)-1-PhEt | Val | NA ^c | > 3 | ND |
| | | | | | | |
| 4e | 1-Nap | BnCH ₂ -CH ₂ | Val | | 1.7 | > 100 |
| 4f | 1-Nap | <i>o</i> -ClBn | Val | 33:67 | 0.43 | > 100 |
| 4g | 1-Nap | <i>m</i> -ClBn | Val | 31, 25, 44 | 0.68 | ND |
| 4h | 1-Nap | <i>p</i> -ClBn | Val | 35:65 | 0.62 | ND |
| 4i | 1-Nap | <i>o</i> -MeBn | Val | 36:64 | 1.0 | 100 |
| 4j | 1-Nap | tBuCH ₂ | Val | NA ^c | 1.5 | > 100 |

^aNA: not available. ND: not determined. ^bSee Table 1. ^cSee Table 3.

amino acids such as the L-leucine derivative **3b** were the most potent EC₅₀ = 120 nM. Branching at the β carbon of the amino acid as in the L-valine and L-isoleucine derivatives **3a** and **3f**, resulted in a 10-fold decrease in activity, however, these ProTides retained sub-μM activity and thus were still more active than **1c**. Compound **3b** was ca. 30 times more active than **1c**.

The plasma stability of these alternate amino acid ProTides was examined in the hope of finding improved rodent plasma stability. The results of testing in mouse plasma for compounds **3a**–**3g** are reported in Table 4. Disubstitution of the α carbon of the amino acid, **3g**, did not noticeably improve mouse plasma stability relative to the L-alanine derivative **2i**. Longer chain amino acids also failed to provide plasma stability. However, branching at the β carbon did provide significantly improved plasma stability. Thus, the Val and Ile derivatives **3a** and **3f** provided 66% and 95% stability in mouse plasma even after 4 h. This stability was measured at 2–4 °C and clearly would be worse at 37 °C. However, this level of stability would at least allow generation of calibration curves and subsequent measurement of plasma ProTide levels in rodents. These branched ProTides maintained their stability in the plasma from other species, but their activity in the replicon assay was somewhat less than that of the L-alanine analogues.

Thus, we subsequently prepared a small family of L-valine analogues with ester variations to examine whether we could improve antiviral activity and maintain rodent plasma stability. The derivatives made are listed in Table 5 and are compared to compound **3a**. Table 5 includes a 2-naphthyl derivative **4a** and the D-valine derivative **4b**, both of which are

Table 6. Plasma Stability of L-Valine Esters

| ProTide | ester | time | % remaining in plasma at 2–4 °C | | | | |
|-----------|--------------------|--------|---------------------------------|------|--------|-----|-------|
| | | | human | cyno | canine | rat | mouse |
| 4a | Bn ^a | 30 min | 93 | 100 | 95 | 49 | 99 |
| | | 4 h | 100 | 95 | 99 | 22 | 76 |
| 4b | Bn ^b | 30 min | 70 | 68 | 69 | 82 | 51 |
| | | 4 h | 82 | 80 | 61 | 56 | 8 |
| 4c | cHx | 30 min | 97 | | 95 | | 95 |
| 4d | (S)-1-PhEt | 30 min | 97 | | 100 | | 100 |
| 4f | <i>o</i> -ClBn | 30 min | 77 | | 100 | | 69 |
| 4g | <i>m</i> -ClBn | 30 min | 100 | | 90 | | 79 |
| 4h | <i>p</i> -ClBn | 30 min | 95 | | 89 | | 94 |
| 4i | <i>o</i> -MeBn | 30 min | 100 | | 100 | | 67 |
| 4j | tBuCH ₂ | 30 min | 91 | | 79 | | 77 |

^a 2-Naphthol derivative. ^b D-Val derivative.

less active in replicon assay. The other ester derivatives, **4c–4j**, are similar in activity to the lead compound **3a**, with simple aliphatic esters perhaps falling off slightly in potency and benzyl esters with an electron withdrawing group ortho, meta, or para, all being somewhat more potent. However, branching at the benzylic position, as in the (*R/S*)-1-phenylethyl derivative **4d**, resulted in a substantial decrease in activity, which was not observed in the L-alanine series.

Plasma stabilities were examined across this series and are listed in Table 6. A number of interesting observations can be made from the rodent plasma stability data. While the 2-naphthol derivative **4a** has similar stability to the 1-naphthol derivative, the D-valine benzyl ester **4b** was observed to be less stable than the L-valine analogue in rodent plasma. The other L-valine esters maintained rodent, and other species, plasma stabilities with the possible exceptions of the ortho chloro and ortho methyl substituted benzyl esters **4f** and **4i**, which showed minor decreases in stability relative to the para-substituted and unsubstituted benzyl esters.

Discussion of in Vitro S9 Stability Studies

Human, dog, cynomolgus monkey, and rat liver and intestinal S9 stability data, as measured by disappearance of parent compound, were obtained for selected compounds (Table 7). Mouse data were not collected because of difficulty in obtaining mouse liver S9 preparations. All samples were run with and without cofactor (NADPH) and expressed as % remaining compound after 60 min incubations. Because NADPH is a cofactor for P450 isozymes, metabolism in the absence of cofactor was considered non-P450 dependent metabolism. We were interested in identifying which species compared closest to human S9 data and which compounds gave the most desirable profile in human intestinal and liver S9 experiments.

Compounds were compared across species mainly by looking at the ratios of liver vs intestinal metabolism as opposed to absolute numbers in each category. When considered this way, it became clear that rat and monkey were reasonable comparators to the human data, but that the dog in vitro data was the outlier. As an example of this, compound **2d** (Table 7) shows more liver cleavage than intestinal cleavage in human, monkey, and rat, but in the dog it is reversed.

Human S9 data were collected for the majority of project compounds but only a portion of the data are reported here. We were looking for compounds with good stability in human intestinal S9 preparations, but with rapid cleavage in liver, in the presence of cofactor. In addition to looking at the ratio between liver and intestinal cleavage, we also paid attention to the overall degree of cleavage. The majority of compounds

Table 7. Multiple Species Liver and Intestinal S9 Data for Selected Compounds with 60 min Incubations (% Remaining)

| ProTide | S9% remaining | human | dog | monkey | rat |
|-----------|---------------|--------------------|-------------------|--------------------|--------------------|
| 2a | liver ± | 20/40 | 67/85 | ND | ND |
| | intestine ± | 60/63 | 50/54 | ND | ND |
| 2d | liver ± | 27/41 | 81/91 | 0/86 | 25/28 |
| | intestine ± | 65/85 | 66/68 | 48/87 | 77/76 |
| 2e | liver ± | 16/50 | 53/80 | ND | ND |
| | intestine ± | 42/48 | 82/100 | ND | ND |
| 2h | liver ± | 7/59 | 49/87 | 16/88 | 5/31 |
| | intestine ± | 28/49 | 42/56 | 20/68 | 57/86 |
| 2i | liver ± | 27/41 ^a | 16/53 | 0/32 ^a | 0/0 ^a |
| | intestine ± | 0/49 ^a | 8/29 | 0/30 ^a | 30/46 ^a |
| 2j | liver ± | 14/29 | 33/28 | ND | ND |
| | intestine ± | 24/33 | 23/27 | ND | ND |
| 3a | liver ± | 0/78 | 56/75 | 0/77 ^a | 8/62 |
| | intestine ± | 24/70 | 56/72 | 25/77 ^a | 65/81 |
| 3b | liver ± | 0/44 ^a | 14/48 | ND | ND |
| | intestine ± | 8/28 ^a | 12/33 | ND | ND |
| 3c | liver ± | 3/14 | 27/40 | ND | ND |
| | intestine ± | 42/59 | 57/86 | ND | ND |
| 3e | liver ± | 0/20 ^a | 4/15 ^a | ND | ND |
| | intestine ± | 0/5 ^a | 0/0 ^a | ND | ND |
| 3f | liver ± | 29/83 | 64/84 | 0/89 ^a | 37/78 |
| | intestine ± | 52/74 | 87/96 | 29/95 | 71/70 |

^a Incubated for 30 min. ± refers to with/without cofactor.

demonstrated greater stability in intestinal S9 preps than in liver, as was desired (Table 7). In this general sense, these compounds could be considered as liver targeting. The lone exception was the benzyl ester of L-alanine, compound **2i**, which was highly cleaved in the intestine. Stability in intestinal cells was considered important because we wanted the ProTide to pass through the gut and intestine and be absorbed and cleaved by the liver. The intestinal stabilities, as measured by % remaining after 60 min incubations, ranged from 0% to 65%. The most stable of this set of compounds was **2d**, the isopropyl ester of L-alanine.

As noted above, the compounds in Table 7 were all incubated with and without cofactor. Looking across the data in the table, there is a trend toward greater cofactor dependence in the liver than in the intestine. Because P450 isozymes require a cofactor, and because P450 levels are high in liver, this is taken as a further subtle indication of liver targeting. The benzyl ester derivatives such as compound **3a** showed a large degree of cofactor dependent liver cleavage, as did the neopentyl ester derivative compound **2h**.

Mouse DMPK Results and Discussion

A series of ester and amino acid derivatives were selected for in vivo studies based on a combination of factors including potency, plasma stability, and SAR. Primarily L-valine derivatives were investigated because they had the most rodent plasma stability. The in vivo work focused on oral dosing not only because it is a desired route of administration in general but also because HCV is a liver disease and absorbed ProTide would pass directly into the liver via the portal vein. For HCV, the general problem of first pass metabolism may work in our favor to produce high levels of the active agent. The overall goal would be to find a compound with low systemic nucleoside exposure but good therapeutic liver triphosphate levels.

For all the ProTides, systemic levels of the parent ProTides were not detectable in the mouse (data not shown). Even for the L-valine derivatives, which demonstrated much improved

Table 8. Mouse Systemic Nucleoside Levels from 50 mg/kg PO Dose of ProTide

| ProTide | C_{\max} ng/mL | C_{last} ng/mL (h) | $T_{1/2}$ h | AUC_{0-t} ng·h/mL | $AUC_{0-\text{inf}}$ ng·h/mL | DNAUC |
|-----------|------------------|-----------------------------|-------------|---------------------|------------------------------|-------|
| 1c | 5203 | 22 (24 h) | 5.4 | 20390 | 20567 | 411 |
| 2h | 262 | 103 (6 h) | 5.2 | 1008 | 1920 | 38 |
| 2n | 1380 | 222 (6 h) | 1.8 | 3722 | 4269 | 85 |
| 3a | 504 | 130 (6 h) | 2.3 | 1611 | 2012 | 40 |
| 4c | 185 | 1.5 (24 h) | 3.0 | 1664 | 1670 | 33 |
| 4f | 518 | 202 (6 h) | 3.5 | 1600 | 2447 | 49 |
| 4g | 388 | 388 (6 h) | | 1650 | | > 33 |
| 4h | 339 | 43 (24 h) | 6.9 | 3990 | 4420 | 88 |
| 4i | 668 | 688 (6 h) | > 6 | 2590 | | 52 |
| 4j | 652 | 244 (6 h) | 3.0 | 2269 | | > 45 |

rodent plasma stability, ProTides were not observed in systemic circulation.

Table 8 reports the plasma levels of parent nucleoside (**1c**) that result from a po dose of ProTide. Mice were orally dosed at 50 mg/kg in an experimental formulation of 5% DMA, 20% Solutol HS 15, 20% PEG 400, 55% 50 mM sodium acetate, pH 4.0. Plasma nucleoside levels were measured at 0.25, 0.5, 1, 2, 4, 6, and 24 h and are compared to the oral PK of the parent nucleoside itself. The parent nucleoside (**1c**) demonstrates only modest bioavailability from a 50 mg/kg oral dose. However, the percent oral bioavailability, calculated from the po and iv (data not shown) DNAUCs values, is quite high ($F = 85\%$). The dose normalized AUC (DNAUC) for the nucleoside **1c** is 411 ng·h/mL/mg/kg, which is higher than the DNAUCs for any of the different ProTides. It should be noted that the oral doses of the different compounds were not molar equivalent doses, so the reported values are not exact comparisons. However, it is clear from the Table 8 data that all the ProTides result in lower systemic exposure of parent nucleoside. The ProTides all have nucleoside DNAUCs that are all 5–10-fold lower than parent nucleoside. This is consistent with the substantially lower nucleoside C_{\max} values that were also observed. It is considered possible that these lower systemic exposures to the parent nucleoside may lead to lower in vivo toxicity.

Comparing the ProTides to each other, the highest nucleoside C_{\max} and DNAUC values belong to the L-alanine derivative **2n**, while the lowest come from the L-valine derivative **4c**. The selection of the ester group also makes a significant difference in C_{\max} and DNAUC. For the two L-alanine derivatives **2h** and **2n**, the (S)-1-phenylethyl ester **2n** resulted in a 5-fold increase in C_{\max} and 2-fold increase in DNAUC over the neopentyl ester **2h**. A similar comparison in the L-valine series is not possible because the (S)-1-phenylethyl ester in the L-valine series was inactive and not tested. Comparison of the neopentyl esters in the L-alanine, and L-valine series shows a higher nucleoside C_{\max} and DNAUC for the L-valine (**4j**) than the L-alanine (**2h**). The benzyl ester derivatives **3a**, **4f**, **4g**, **4h**, and **4i**, were the most potent compounds in the L-valine series. These compounds along with the primary alcohol ester **4j**, produced the highest C_{\max} values for parent nucleoside. Interestingly, the secondary alcohol derivative **4c** has a much lower C_{\max} and over all nucleoside exposure than the other L-valine esters.

Although the very potent neopentyl L-alanine derivative **2h** showed low systemic levels of nucleoside, as was desired, because of their inherent rodent plasma instability, the L-alanine derivatives were not further studied in the mouse. Several of the L-alanines were later studied in cynomolgous monkeys, and their data will be reported elsewhere.

Although maintaining low systemic nucleoside levels is desirable, it only makes sense if sufficient liver triphosphate

Table 9. Comparison of 2'-C-Methyl Guanosine Triphosphate Liver Levels from 50 mg/kg PO Dose of ProTide

| ProTide | C_{\max} ng/g (h) | C_{last} ng/mL (h) | AUC_{0-6} h·ng/g | AUC_{0-24} h·ng/g | DNAUC |
|-------------------|---------------------|-----------------------------|--------------------|---------------------|-------|
| 1c | 3470 (2) | 390 (24) | 16510 | 36868 | 737 |
| 2h | 370 (6) | 370 (6) | 1584 | | 32 |
| 2n , no. 1 | 1950 (4) | 1158 (6) | 6980 | | 140 |
| 2n no. 2 | 670 (4) | 134 (16) | 2596 | 5390 | 108 |
| 3a | 512 (4) | 470 (6) | 1940 | | 97 |
| 4c | 275 (4) | 74 (24) | 815 | 3860 | 77 |
| 4f | 290 (6) | 290 (6) | 880 | | 18 |
| 4g | 216 (6) | | | | |
| 4h | 225 (4) | 166 (6) | 920 | | 18 |
| 4i | 91 (6) | | | | |
| 4j | | | | | |

levels are achieved. Table 9 reports the 2'-C-methyl guanosine triphosphate levels in mouse liver. Liver levels were determined at 0.25, 0.5, 1, 2, 4, and 6 h. For three compounds, **1c**, **4c**, and **4h**, the 24 h liver levels were also measured. The NTP DNAUC values were calculated based on actual values not projections to 24 h. This results in significantly lower DNAUC values for several of the compounds with only 6 h data points. Once again, these doses are nonequivalent on a molar basis, and as a result the triphosphate levels of **1c** are approximately 2-fold higher than if dosed at an equivalent level.

As mentioned above, we have observed the nucleoside **1c** to have excellent oral bioavailability in the mouse ($F = \sim 85\%$). It is rapidly absorbed, and its metabolism in mouse liver is modest, leading to similar exposure from an oral dose as from an iv dose. On the other hand, oral bioavailability in the cynomolgus monkey is poor ($F \sim 10\%$, data not shown). Clearly, there are major differences in how the mouse and nonhuman primates absorb and metabolize 2'-C-methylguanosine and its ProTides, and the mouse may not be an appropriate model for studying 2'-C-methylguanosine ProTide pharmacokinetics. However, liver triphosphate levels can be measured in the mouse at multiple time points so very valuable data can be obtained.

The liver triphosphate levels from the parent nucleoside in the mouse were substantially higher than those from the ProTides, mimicking the systemic nucleoside levels. Even accounting for dosing differences, and extrapolating out the 6 h DNAUC values to 24 h, all ProTides provide 2–5-fold less liver triphosphate, as measured by DNAUC, than does the nucleoside itself.

However, in considering the ratio of the triphosphate DNAUC to the nucleoside DNAUC, several ProTides have similar, to perhaps better, ratios than the nucleoside **1c**. The ProTides **2n**, **4a**, and **2c** have triphosphate DNAUC to nucleoside DNAUC ratios of about 2, similar to **1c**. For the L-alanine derivative **2h**, or the L-valine derivative **3a**, the DNAUC values are calculated only using 6 h data. If the DNAUC values are

extrapolated out to 24 h, it is quite possible that the triphosphate to nucleoside ratio for these two compounds would be better than that obtained for the nucleoside.

The best overall triphosphate exposure appears to come from the (*S*)-1-phenyl ethyl ester of L-alanine phosphoramidate (**2n**). This compound was examined in an additional experiment out to 16 h. A certain amount of interexperiment variation was observed due to the extreme difficulty of measuring triphosphate levels in the liver. The DNAUC from the second experiment was 140 ng·h/mL/mg/kg. Nucleoside systemic exposure was also measured in the second experiment, and the DNAUC for nucleoside was 71 ng·h/mL/mg/kg.

In summary of the mouse DMPK work, the ProTides produce significantly lower liver triphosphate levels than the parent nucleoside, however, several compounds could be identified with improved ratios of liver triphosphate to systemic nucleoside levels. The mouse is considered a nonoptimal model for studying ProTides of 2'-*C*-methyl guanosine because of the observed rapid breakdown of the ProTides in the mouse and because of the good absorption and metabolic stability of the resulting 2'-*C*-methyl guanosine. However, very useful data were obtained comparing different ProTides to each other and parent nucleoside. Further work on ProTides and other pro-drugs of 2'-*C*-methyl guanosine is underway in the cynomolgus monkey, and will be reported elsewhere.

Conclusions

We have reported herein an extensive ProTide study of 2'-*C*-methylguanosine, with variations in the aryl, ester, and amino acid region of the ProTide. In almost every case, the ProTides are more active in the HCV replicon assay, than the parent nucleoside, often by 10–30-fold. We have extensively studied the compounds for stability in plasma from multiple species and also in liver and intestinal preparations from several species. A combination of ester and amino acid changes gives rodent plasma-stable compounds. Several analogues were further evaluated for oral PK in mouse and reveal rapid uptake and metabolism to triphosphate in the liver. The data are somewhat suggestive of liver targeting by these ProTides. This, and their inherent potency and selectivity against HCV *in vitro*, suggests that their continued preclinical evaluation is warranted.

Experimental Section

General. Anhydrous solvents were purchased from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with ³¹P NMR spectra. Column chromatography was performed on silica gel (35–70 μM). Proton (¹H), carbon (¹³C), and phosphorus (³¹P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak and all ¹³C NMR, ³¹P NMR were proton-decoupled. Analytical and semipreparative HPLC were conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector) using Varian Polaris C18-A (10 μM) as an analytic column and Varian Polaris C18-A (10 μM) as a semipreparative column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient (system 1, 100/0 to 0/100 v/v in 30 min) or water/methanol (system 2, 100/0 to 0/100 v/v in 30 min). High resolution mass spectra was performed as a service by Cardiff University, using electrospray (ES). CHN microanalysis were performed as a service by the School of Pharmacy at the University of London

and by MEDAC Ltd., Surrey. 2'-*C*-Methyl guanosine 5'-triphosphate was purchased from Carbosynth, Berkshire, UK.

Compound purity was assured by a combination of high field multinuclear NMR (H,C,P) and HPLC. Purity by the latter was always >95% with no detectable parent nucleoside, for all final products.

Replicon Potency. Huh7 cells expressing the HCV genotype 1b bicistronic subgenomic replicon (Apath, LLC, Brooklyn, NY) were seeded into white 96-well plates (Nunc/VWR) at a density of 2×10^4 cells/well in medium without G-418. A Stacker multidrop liquid dispenser (MTX Lab Systems, Vienna, VA) was employed to ensure uniform and fast cell seeding into multiple plates. Then 18–24 h after cell plating, inhibitors were added and cells were incubated for additional 48 h. Compounds were tested in triplicates and quadruplicates at $3 \times$ or $4 \times$ serial dilutions over a range of 0.0001–10 μM concentrations. HCV replication was monitored by Renilla luciferase reporter activity assay using Renilla luciferase reporter (Promega, Madison, WI) and a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). Then 50% inhibitory concentration (EC₅₀) values were calculated as the concentration of compound that results in 50% decrease in the reporter expression as compared to untreated cells. The values were determined by nonlinear regression (four-parameter sigmoidal curve fitting) analysis. For any one IC₅₀ determination, the replicon assay was run in triplicate and standard deviation was calculated from the three repeats and this standard deviation is usually less than 20% of the IC₅₀. A second standard deviation is determined when multiple IC₅₀s are obtained from different batches of replicon cells. When more than three independent IC₅₀ values have been obtained, this second standard deviation is calculated. Because of the differences in the different batches of replicon cells, this standard deviation is often ca. 100% of the IC₅₀.

Cytotoxicity Assays. The same cells used in the replicon assay were seeded into 96-well plates at a density of 2×10^4 cells per well. Twenty-four h after cell plating, 11 serial $2 \times$ compound dilutions, starting with 100 μM, were applied to the testing plates (three repeats per compound dilution). Each testing plate was run with a “no-compound” control. Incubation with compounds was continued at 37 °C in a CO₂ incubator for 72 h. The cells were in log phase of growth throughout the cell cytotoxicity experiments. To determine cell viability, the CellTiter-Glo assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. The compound concentration resulting in 50% luminescent signal was reported as the CC₅₀ concentration.

Plasma Stability. Stability experiments were performed in duplicate using human, cynomolgus monkey, canine, rat, and mouse plasma (Bioreclamation, Inc., Long island, NY). ProTides were added to a final concentration of 1 μg/mL in 1 mL of plasma preincubated at 2–4 °C. The reaction mixture was maintained at 2–4 °C, and 50 μL samples were taken at 30 or 240 min of incubation and transferred to a 96-well V-bottom plate. Then 200 μL of ice cold acetonitrile was added to each sample. The precipitated samples were centrifuged at 2500 rpm, 4 °C, for 20 min in a Sorvall RT6000S centrifuge (Thermo Scientific, Waltham MA). Then 50 μL of supernatant from each sample was transferred to a 96-deep well plate followed by the addition of 50 μL of H₂O to each sample. Samples were covered, mixed well by vortexing, and maintained at 2–8 °C before and during analysis. Fifteen μL of each test sample was analyzed for compound concentrations by LC-MS/MS. Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Synergi 4 μm Polar-RP, 30 mm \times 2.0 mm column (Phenomenex, Torrance, CA). Linear gradient (100% mobile phase A (H₂O + 0.1% HCOOH) to 100% B (acetonitrile + 0.1% HCOOH) over 3 min, flow rate 1.0 mL/min) was used for the analyte elution. The HPLC system was coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectrometry analysis was performed in positive ion mode (MH⁺ 659.2, transition 166.1).

Data was analyzed using Analyst v1.4.2 software (Applied Biosystems, Framingham, MA), Microsoft Office Excel 2007, and GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

S9 Clearance Assay. All liver and intestinal S9 tissue fractions were purchased from Xenotech (Lenexa, KS) and stored at -80°C until time of use. NRS (NADPH-Regenerating System) was purchased from BD Gentest (BD Biosciences, San Jose, CA) and was used as cofactor. Reagents including NRS components and liver or intestinal S9 tissue fractions were thawed and immediately placed on ice. S9 fractions, 50 mM potassium phosphate buffer with or without NRS were placed in wells of a 96-well deep-well plate for final concentrations of 1 mg/mL S9, 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride. The plate was preincubated in an orbital shaker at 37°C for 10 min. The reaction was then initiated by addition of compound at a final concentration of 10 μM . The suspensions were thoroughly mixed by pipetting and an initial sample was withdrawn and added to equal volume of cold acetonitrile (for a calculation of starting concentration). The plate was placed back in the 37°C shaker, and at 60 min a sample was withdrawn and added to equal volume cold acetonitrile. Following quenching by acetonitrile, samples were kept on ice and precipitate was pelleted in all samples by centrifugation at 3000 rpm for 15 min. Supernatant was removed and transferred to vials for HPLC analysis.

Pharmacokinetic Studies. Compounds were formulated in 5% dimethylacetamide, 20% Solutol HS15, 20% polyethylene glycol 400, 55% 50 mM sodium acetate, (pH 4) (D/S/P) and administered by oral gavage to female ICR mice (Taconic Farms, Germantown, NY). Blood and tissue collections were performed at various time points as terminal procedures. Blood was collected into EDTA containing tubes (1.6 mg EDTA/mL blood, Sarstedt, Inc., Newton, NC), and the plasma was separated by centrifugation within 30 min of collection. Liver samples were immediately frozen upon collection in liquid nitrogen. Plasma and liver samples were stored frozen at $\leq -80^{\circ}\text{C}$ prior to analysis.

Measurement of Plasma Nucleoside Concentrations. Plasma samples were prepared for analysis as follows. First, 400 μL of 50 mM ammonium acetate was added to 100 μL of each plasma sample. Calibration curves were prepared by serial dilution of a stock solution of 2'-C-methyl guanosine (**1c**) into control plasma. Solid phase extraction of the samples was performed with H₂O-Phylic speeddisk columns (J.T. Baker) which were previously solvated with 1 mL of methanol followed by equilibration with 1 mL of 50 mM ammonium acetate. The columns were rinsed with 1 mL of 50 mM ammonium acetate:methanol (95:5, v/v), and samples were eluted with 1 mL of methanol:ammonium hydroxide (95:5, v/v). The samples were dried under nitrogen and reconstituted in 80 μL of H₂O. A volume of 10 μL of each test sample was analyzed for 2'-C-methyl guanosine (**1c**) concentrations by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Betasil 2.1 mm \times 100 mm 5 μm column (Thermo Scientific). Mass spectrometry analysis was in positive ion mode as described above.

Measurement of Liver Triphosphate Concentrations. Liver samples were snap-frozen in liquid nitrogen and pulverized with a steel mortar and pestle on dry ice with liquid nitrogen. Frozen, pulverized tissue samples were weighed and 5 equiv of 70% methanol containing 50 mM thenoyltrifluoroacetone was added. Samples were then homogenized twice in a bead mill (FastPrep Homogenizer) using Silicone-Carbide Sharp Particles (BioSpec Products, Inc., Bartlesville, OK) at 4°C at an agitating speed of 6 for 30 s. The homogenates were centrifuged at 15000 rpm for 30 min at 4°C . The supernatants were collected and dried in Masterblock polypropylene deep-well plates (SPEware Corp., Baldwin Park, CA) under a stream of nitrogen. The dried extracts

were reconstituted with 100 μL of 10 mM *N,N*-dimethylhexylamine, 3 mM ammonium formate in H₂O, vortexed, and centrifuged at 3500 rpm for 30 min at 4°C . Calibration curves were constructed by spiking varying concentrations of 2'-C-methyl guanosine 5'-triphosphate (Carbosynth, Berkshire, UK) into control liver samples prior to homogenization. Fifteen μL of each test sample was analyzed for 2'-C-methyl guanosine 5'-triphosphate concentrations by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Reverse phase liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with an XTerra MS C18, 3.5 μm , 2.1 mm \times 50 mm Column (Waters, Milford, MA). Mass spectrometry analysis was performed in negative ion mode and data were analyzed as above. The levels (peak areas) of endogenous adenosine 5'-triphosphate and guanosine 5'-triphosphate were also monitored as internal quality controls for the liver samples.

Pharmacokinetic Analysis. Plasma and liver concentration data was analyzed for standard pharmacokinetic parameters using Win-Nonlin v5.2 software (Pharsight, St. Louis, MO).

Standard Procedure A: ProTide Synthesis. To the 2',3'-*O*,*O*-isopropylidene-2'-C- β -methylguanosine (1 equiv) in anhydrous THF (6 mL/g of nucleoside) was added dropwise a solution of *t*BuMgCl in THF (1M, 2 equiv). After stirring for 20 min, a solution of the phosphorochloridate (2 equiv) in anhydrous THF (6 mL/g of phosphorochloridate) was slowly added. The resulting solution was stirred overnight at room temperature, then the solvent was removed under reduced pressure. The resulting foamy residue was purified by silica gel column chromatography (eluent: CHCl₃/MeOH 92:8, v/v) to afford the pure protected ProTide.

Standard Procedure B: ProTide Deprotection. A solution of the protected ProTide in a 60% acetic acid in water solution (~ 10 mL/200 mg of protected protide) was stirred at 95°C overnight. The solvent was then removed under reduced pressure to dryness (can also be coevaporated with hexane or toluene to remove traces of AcOH), and the resulting residue was purified by silica gel column chromatography (eluent: CHCl₃/MeOH 9:1, v/v) to give after lyophilization the pure ProTide.

Example: Synthesis of 2'-C-Methylguanosine-5'-*O*-(naphthyl(methoxy-*L*-alaninyl))phosphate (2a**).** Step 1: Synthesis of the protected ProTide

Prepared using standard procedure A. Starting from 181 mg of protected nucleoside. Yield: 37%.

³¹P NMR (202 MHz, CD₃OD) δ 4.49, 4.40. ¹H NMR (500 MHz, CD₃OD) δ 8.17 (2d, $J = 8.0$, 1H, H₈-napht), 7.85 (m, 2H, H₅-napht and H₈), 7.70 (m, 1H, H₄-napht), 7.58–7.48 (m, 3H, H₇, H₆, H₂-napht), 7.42 (m, 1H, H₃-napht), 6.08 and 6.07 (s, 1H, H_{1'}), 4.62–4.54 (m, 2H, H_{3'} and H_{5'}), 4.48–4.36 (m, 2H, H_{4'} and H_{5''}), 4.09 (m, 1H, H α), 3.61 (s, 3H, CH₃ ester), 1.59 and 1.57 (2s, 3H, CH₃ isopropylidene), 1.36 (m, 6H, CH₃ isopropylidene and CH₃ Ala), 1.03 and 1.00 (s, 3H, 2'-CH₃).

Step 2: Deprotection of the ProTide

Prepared using standard procedure B. Starting from 70.5 mg. Yield: 45% (30 mg).

³¹P NMR (202 MHz, CD₃OD) δ 4.36, 4.27. ¹H NMR (500 MHz, CD₃OD) δ 8.18 (m, 1H, H₈-napht), 7.93–7.83 (m, 2H, H₅-napht and H₈), 7.69 (m, 1H, H₄-napht), 7.56–7.49 (m, 3H, H₇, H₆, H₂-napht), 7.40 (m, 1H, H₃-napht), 5.94 (s, 1H, H_{1'}), 4.57 (m, 2H, H_{3'} and H_{5''}), 4.29–4.23 (m, 2H, H_{4'} and H_{3'}), 4.10–3.98 (m, 1H, H α), 3.56 and 3.55 (2s, 3H, CH₃ ester), 1.30 (m, 3H, CH₃ Ala), 1.01 and 0.99 (2s, 3H, 2'-CH₃). HPLC, system 1, RT 13.00, 13.43 min.

Acknowledgment. We thank Helen Murphy for secretarial assistance.

Supporting Information Available: Preparative methods, spectroscopic and analytical data on target compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Hoofnagle, J. H.; Seef, L. B. Peginterferon and ribavirin for chronic hepatitis C. *N. Engl. J. Med.* **2006**, *355*, 2444–2451.
- (2) Soriano, V.; Madejon, A.; Vispo, E.; Labarga, P.; Garcia-Samaniego, J.; Martin-Carbonero, L.; Sheldon, J.; Bottecchia, M.; Tuma, P.; Barreiro, P. Emerging drugs for hepatitis C. *Expert Opin. Emerging Drugs* **2008**, *13*, 1–19.
- (3) Brown, N. A. Progress towards improving antiviral therapy for hepatitis C with hepatitis C virus polymerase inhibitors. Part 1: Nucleoside analogues. *Expert Opin. Invest. Drugs* **2009**, *18*, 709–725.
- (4) Klumpp, K.; Leveque, V.; Le Pogam, S.; Ma, H.; Jiang, W.-R.; Kang, H.; Granycome, C.; Singer, M.; Laxton, C.; Hang, J. Q.; Sarma, K.; Smith, D. B.; Heindl, D.; Hobbs, C. J.; Merrett, J. H.; Symons, J.; Cammack, N.; Martin, J. A.; Devos, R.; Najera, I. The novel nucleoside analog R1479 (4'-azidocytidine) is a potent inhibitor of NS5B-dependent RNA synthesis and hepatitis C virus replication in cell culture. *J. Biol. Chem.* **2006**, *281*, 3793–3799.
- (5) Eldrup, A. B.; Prhavc, M.; Brooks, J.; Bhat, B.; Prakash, T. P.; Song, Q.; Bera, S.; Bhat, N.; Dande, P.; Cook, P. D.; Bennett, C. F.; Carroll, S. S.; Ball, R. G.; Bosserman, M.; Burlein, C.; Colwell, L. F.; Fay, J. F.; Flores, O. A.; Getty, K.; LaFemina, R. L.; Leone, J.; MacCoss, M.; McMasters, D. R.; Tomassini, J. E.; Langen, D. V.; Wolanski, B.; Olsen, D. B. Structure–Activity Relationship of heterobase modified 2'-C-methyl ribonucleosides as inhibitors of hepatitis C virus RNA replicaton. *J. Med. Chem.* **2004**, *47*, 5284–5297.
- (6) Cahard, D.; McGuigan, C.; Balzarini, J. Aryloxy phosphoramidate Triesters as Pro-Tides. *Mini-Rev. Med. Chem.* **2004**, *4*, 371–382.
- (7) McGuigan, C.; Cahard, D.; Sheeka, H. M.; De Clercq, E.; Balzarini, J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of a novel intracellular metabolite. *J. Med. Chem.* **1996**, *39*, 1748–1753.
- (8) McGuigan, C.; Kelleher, M. R.; Perrone, P.; Mulready, S.; Luoni, G.; Daverio, F.; Klumpp, K.; Smith, D. B. The application of phosphoramidate ProTide technology to the potent anti-HCV aompound 4'-azidocytidine. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4250–4254.
- (9) McGuigan, C.; Perrone, P.; Madela, K.; Neyts, J. The phosphoramidate ProTide approach greatly enhances the activity of beta-2'-C-methylpurines against Hepatitis C Virus. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4316–4320.
- (10) Furman, P. A.; Wang, P. Y.; Niu, C. R.; Bao, D. H. Symonds, W.; Nagarathnam, D.; Steuer, H. M.; Rachakonda, S.; Ross, B. S.; Otto, M. J.; Sofia, M. PSI-7851: A novel liver-targeting nucleotide prodrug for the treatment of Hepatitis C. *Hepatology* **2009**, *48* (Suppl), presented at 59th Annual Meeting of the American Association for the Study of Liver Diseases, Oct 31–Nov 4, 2008, San Francisco, CA, poster 1901.
- (11) Birkus, G.; Kutty, N.; He, G.-X.; Mulato, A.; Lee, W.; McDermott, M.; Cihlar, T. Activation of GS-7340 and other Tenofovir phosphonoamidate prodrugs by human proteases. *Antiviral Res.* **2007**, *74*, A57.
- (12) Notably, the agent has recently been resolved into its separate diastereoisomers. See: Chun, B. K.; Rachakonda, S.; Du, J. F.; Khan, N.; Shi, J. X.; Stee, W.; Cleary, D.; Ross, B. S.; Sofia, M. J. An efficient and diastereoselective synthesis of PSI-6130: a clinically efficacious inhibitor of HCV NS5B Polymerase. *J. Org. Chem.* **2009**, *74*, 6819–6824.
- (13) McGuigan, C.; Sutton, P. W.; Cahard, D.; Turner, K.; O'Leary, G.; Wang, Y.; Gumbleton, M.; De Clercq, E.; Balzarini, J. Synthesis, anti-human immunodeficiency virus activity and esterase lability of some novel carboxylic ester-modified phosphoramidate derivatives of stavudine (d4T). *Antivir. Chem. Chemother.* **1998**, *9*, 473–479.
- (14) Zhou, X. J.; Pietropaolo, K.; Sullivan-Bolyai, J.; Kuca, B.; Liu, W.; Xu, L.; Belanger, B.; Khan, S.; Mayers, D. IDX184, A liver-targeted nucleotide HCV polymerase inhibitor: Results of a first-in-man safety and pharmacokinetic study. Presented at EASL, 25th April 2009, poster no. 966.
- (15) Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. The application of phosphoramidate ProTide technology to Acyclovir confers anti-HIV inhibition. *J. Med. Chem.* **2009**, *52*, 5520–5530.
- (16) ClogP values were calculated using ChemOffice Ultra 11.0.
- (17) McGuigan, C.; Tsang, H. W.; Cahard, D.; Turner, K.; Velazquez, S.; Salgado, A.; Bidois, L.; Naesens, L.; De Clercq, E.; Balzarini, J. Phosphoramidate derivatives of d4T as inhibitors of HIV: the effect of amino acid variation. *Antiviral Res.* **1997**, *35*, 195–204.



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus

Christopher McGuigan^{a,*}, Karolina Madela^a, Mohamed Aljarah^a, Arnaud Gilles^a, Andrea Brancale^a, Nicola Zonta^a, Stanley Chamberlain^b, John Vernachio^b, Jeff Hutchins^b, Andrea Hall^b, Brenda Ames^b, Elena Gorovits^b, Babita Ganguly^b, Alexander Kolykhalov^b, Jin Wang^b, Jerry Muhammad^b, Joseph M. Patti^b, Geoffrey Henson^b

^a Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK

^b Inhibitex Inc, 9005 Westside Parkway, Alpharetta, GA 30009, USA

ARTICLE INFO

Article history:

Received 13 May 2010

Revised 16 June 2010

Accepted 17 June 2010

Available online 20 June 2010

Keywords:

HCV

ProTide

Phosphoramidate

Nucleoside

Nucleotide

Antiviral pro-drug

Polymerase

NS5B

ABSTRACT

We herein report a novel double pro-drug approach applied to the anti-HCV agent 2'-β-C-methyl guanosine. A phosphoramidate ProTide motif and a 6-O-methoxy base pro-drug moiety are combined to generate lipophilic prodrugs of the monophosphate of the guanine nucleoside. Modification of the ester and amino acid moieties lead to a compound INX-08189 that exhibits 10 nM potency in the HCV genotype 1b subgenomic replicon, thus being 500 times more potent than the parent nucleoside. The potency of the lead compound INX-08189 was shown to be consistent with intracellular 2'-C-methyl guanosine triphosphate levels in primary human hepatocytes. The separated diastereomers of INX-08189 were shown to have similar activity in the replicon assay and were also shown to be similar substrates for enzyme processing. INX-08189 has completed investigational new drug enabling studies and has been progressed into human clinical trials for the treatment of chronic HCV infection.

© 2010 Elsevier Ltd. All rights reserved.

Over 180 million people are chronically infected with hepatitis C virus (HCV) and at risk of developing life threatening liver disease.¹ The current therapy consists of pegylated interferon and ribavirin.² Neither agent is specific for HCV, side effects are common, and efficacy is limited in certain genotypes.² As with antivirals in general, nucleoside analogues are amongst the leading classes of compounds being developed as new agents for the treatment of HCV infection. Several 2'-C-ribonucleoside analogues, including 2'-C-methyladenosine and 2'-C-methyl guanosine, (**1**) have been shown to possess activity against HCV in the replicon assay as well as antiviral activity against several members of the *Flavivirus* family.^{3,4} 2'-C-methyl guanosine was evaluated further in a series of nonclinical studies, which indicated the absence of detectable cytotoxicity, potent inhibition of the HCV RNA-dependent RNA polymerase as its triphosphate, and oral bioavailability in rats of 82%.^{5,10} Unfortunately, its potential as a therapeutically useful nucleoside was limited due to low oral bioavailability in non-rodent species, inefficient cellular uptake and poor intracellular metabolism of 2'-C-methyl guanosine to its active triphosphate

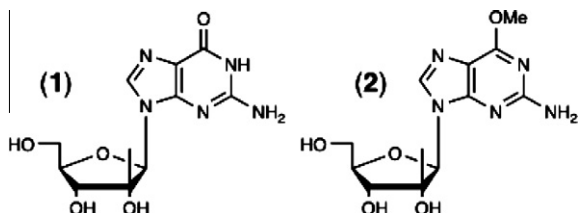
form.⁵ We have previously reported the application of our ProTide, phosphoramidate pro-drug approach⁶ to 2'-C-methyl guanosine (**1**) to overcome these limitations.^{7,8} In this publication we describe a series of novel double pro-drugs of 2'-C-methyl guanosine for HCV therapy.

The HCV antiviral activities of our phosphoramidates were evaluated against HCV genotype 1b, in a Huh7 cell line expressing a stable, bicistronic subgenomic replicon encoding the *Renilla* luciferase reporter gene.⁹ HCV replication in this cell line was monitored by measuring the luminescence produced by luciferase activity. From the initial series of compounds, the naphthyl benzyl-alanine phosphoramidate of (**1**), in the assay described above, is active at 0.062 μM, being ca. 16 times more active than (**1**) which has an EC₅₀ of 1.0 μM. However, subsequent work to address the rodent plasma instability of these compounds lead to L-valine phosphoramidate derivatives of (**1**) such as the naphthyl benzyl L-valine phosphoramidate, which demonstrated much improved rodent plasma stability.⁸ Unfortunately, with the improved rodent plasma stability of the branched amino acids, came a significant decrease in HCV replicon activity. Extensive modification of the ester functionality did not improve HCV potency significantly.⁸ We then turned to modifications of the purine base as a means of

* Corresponding author. Tel./fax: +44 2920874537.

E-mail addresses: mcguigan@cf.ac.uk, mcguigan@cardiff.ac.uk (C. McGuigan).

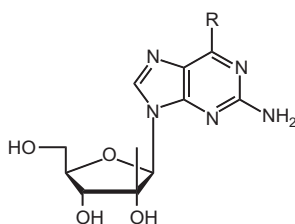
potentially affecting potency without changing the inherent plasma stability of the L-valine phosphoramidates. Modifications were made at the C-6 and C-2 positions using an HCV polymerase model as a guide.¹⁰ SAR development is underway at the C-2 position and will be discussed elsewhere. We considered whether simple C-6 modifications could maintain binding of the corresponding triphosphate to HCV RNA polymerase, and in particular, whether a 6-O-methoxy substituent as in **2** could be tolerated in the model. It was considered that the likely increase in lipophilicity of (**2**) could enhance the poor cell uptake of (**1**).



To test this, an HCV polymerase model was built according to the literature¹⁰ and docking studies with the phosphorylated forms of various C-6 substituted derivatives were performed. These studies showed that the triphosphate of nucleoside **2**, docks only poorly into the NS5b active site, suggesting that it would be a poor inhibitor of the NS5B polymerase.¹¹ The replicon activity of 6-O-methyl-2'-C-methyl guanosine (**2**) was determined and is reported, for the first time, in Table 1 along with the modelling results and replicon activity of a number of other C-6 substituted analogues. The modest replicon activity observed for these derivatives may be ascribed to their slow intracellular conversion to 2'-C-methyl guanosine by deaminase activity, which might be at the nucleoside level (e.g., ADA, EC 3.5.4.4),¹² or at the nucleotide level.

Table 1
Comparison of modelling predictions of the nucleoside triphosphate and HCV replicon activity of C-6 substituted derivatives of 2'-C-methyl guanosine

| R | Modelling prediction | Replicon activity (μM) |
|------------------|----------------------|-------------------------------------|
| OH | Good binding | 1 |
| OCH ₃ | Poor binding | 5 |
| OE _t | Poor binding | 9 |
| SMe | Poor binding | 11 |
| NHMe | Poor binding | 13 |
| NHBn | Poor binding | 27 |
| Cl | Poor binding | 8 |



Compounds in Table 1 were synthesized from the C-6 chloro 2-amino 2'-C-methyl purine riboside, and phosphoramidate derivatives were made of each. Full details of this work will be reported elsewhere.

In spite of the reduced replicon activity of 6-O-methyl-2'-C-methyl guanosine, and the prediction from the modelling that the corresponding triphosphate would be inactive, we sought to prepare a series of ProTides of (**2**) to investigate the effect of the phosphorylation by-pass strategy on the replicon activity of this modified purine. The hope was that phosphoramidates of **2** would show improved cellular uptake, and would be metabolically converted to the active 2'-C-methyl guanosine triphosphate.¹³

Target compound (**2**) was prepared in an overall yield of 60% via the 6-chloro nucleoside generated by the TMS triflate mediated condensation of the tetrabenzoyl 2-C-methyl sugar and chloro base (Fig. 1).

Compound (**2**) was converted to 5'-ProTides following our established methods.¹⁴ In brief, 1-naphthol and POCl₃ were reacted to generate the naphthyloxy phosphorodichloridate and this was allowed to react with various amino acid ester salts to generate the phosphorochloridates (**3**) as key synthons. As shown in Figure 2, reaction of (**3**) with nucleoside (**2**) in THF in the presence of *N*-methyl imidazole gave the target compounds (**4a–m**) in moderate yield. Notably, use of the 6-O-methylated nucleoside as opposed to the guanine nucleoside, allows coupling with the chlorophosphoramidate to proceed without prior protection of the nucleoside sugar hydroxyl groups, saving a deprotection step in the linear synthetic sequence and saving two steps in the overall synthesis.

Compounds (**4**) were purified by flash column chromatography and HPLC as necessary. They were routinely isolated as roughly 1:1 mixtures of phosphate diastereoisomers as evidenced by splitting of HPLC peaks and ³¹P NMR signals. Compounds were tested as mixtures of diastereomers in the first instance. ¹³C NMR and mass spectrometry data also confirmed the structure and purity of (**4a–m**).¹⁵ Compounds (**4a–h**), being the alanine series, were evaluated versus HCV in replicon assay, with data shown in Table 2.

Thus, in general the data in Table 2 show a significant increase in the cell based potency from this family of ProTides, in comparison to the parent nucleoside (**2**). The most active ester is the neopentyl (**4g**) with EC₅₀ of 0.01 μM and EC₉₀ of 0.04 μM . This is ca. 500–550-fold more active than the parent nucleoside (**2**). Notably, comparing the Ala benzyl ester ProTide of (**2**), compound **4a**, with its equivalent ProTide of the guanine parent (**1**)⁷ shows a ca. fourfold potency boost for the 6-methoxy analogue. In part, this may be due to the enhanced lipophilicity and consequent cellular uptake for **4a**; calculated C log *P* values are 3.1 and 1.9, respectively. The much reduced activity of the isopropyl ester derivative (**4c**) highlights the importance of synthesizing multiple phosphoramidate derivatives.

Given the high potency of the neopentyl ester **4g** of the 6-methoxy nucleoside **2**, we also prepared the equivalent ProTide of **1**. The data on this compound, **5** are presented alongside **4g** in Table 3.

Thus, the 6-methoxy analogue shows a calculated lipophilicity some 100 times that of the guanine parent. This translates into a ≥ 5 -fold enhancement in membrane transport as measured by Caco-2 permeation and a >5 -fold boost in HCV potency. This supports the idea that phosphoramidates of **2** have improved cellular uptake over phosphoramidates of **1**.

To pursue this family of phosphoramidates further, we embarked on selective amino acid variation, while retaining the neopentyl ester of the lead (**4g**). Data are shown in Table 4.

From the data in Table 4, it is clear that L-Ala (**4g**) is strongly (24 \times) preferred over D-Ala (**4i**). This highlights the importance of intracellular metabolism in the activity of these phosphoramidates because both have very similar C log *P* values. Increasing the overall size of the amino acid side chain as for the L-Met (**4j**) and L-Leu (**4k**) derivatives decreases HCV activity somewhat, but the most dramatic decrease in activity comes with branching at the amino acid beta carbon as in L-Ile (**4l**) and L-Val (**4m**). Overall, the 6-O-methyl modification consistently improves the HCV replicon activity relative to the guanine derivatives for the different amino acid derivatives in Table 4. For example, the corresponding guanine version of the L-Val derivative, **4m**, is 10-fold less active (EC₅₀ = 1.5 μM) in the replicon assay than is the 6-O-methyl L-Val derivative.⁸

From this survey of amino acid and ester variations, the neopentyl alanine ProTide (**4g**) emerged as one of the more interesting compounds. To further characterize **4g** as a lead compound and to more fully define its potency, it was repeated multiple times in the HCV replicon assay. As shown in Figure 3, replicate assays revealed a

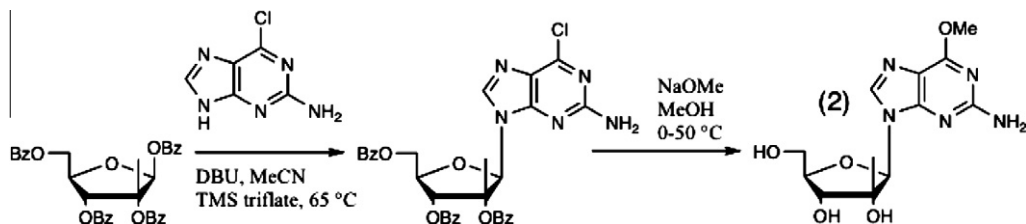


Figure 1. Synthesis of 6-OMe nucleoside (2).

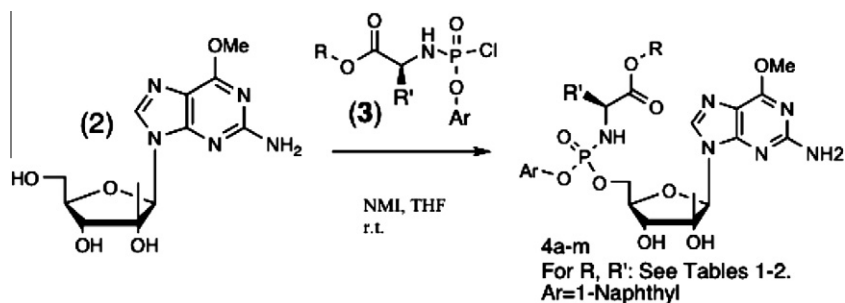


Figure 2. Synthesis of ProTides of (2).

Table 2

Anti-HCV activity (EC_{50} , EC_{90}) and cell cytotoxicity (CC_{50}) of alanine ProTides of (2)

| Compd | R | Genotype 1b replicon | | Huh7 CC_{50} (μ M) |
|-------|---|----------------------|----------------------|---------------------------|
| | | EC_{50} (μ M) | EC_{90} (μ M) | |
| 4a | Bn | 0.03 | 0.10 | 12 |
| 4b | <i>n</i> -Pr | 0.03 | 0.10 | 13 |
| 4c | <i>i</i> -Pr | 0.54 | 1.70 | >100 |
| 4d | cHex | 0.03 | 0.09 | 6 |
| 4e | cPnt | 0.03 | 0.08 | 9 |
| 4f | S-PhEt | 0.04 | 0.13 | 14 |
| 4g | <i>t</i> -BuCH ₂ | 0.01 | 0.04 | 7 |
| 4h | <i>t</i> -BuCH ₂ CH ₂ | 0.02 | 0.08 | 14 |
| 2 | — | 5.2 | 21 | >100 |

Table 3

Anti-HCV activity, cytotoxicity, calculated lipophilicity and observed Caco-2 permeation for 4a and 5

| Compd | Replicon | | CC_{50} (μ M) | C log P^a | Caco-2% ^b |
|-------|----------------------|----------------------|----------------------|-------------|----------------------|
| | EC_{50} (μ M) | EC_{90} (μ M) | | | |
| 4g | 0.010 | 0.038 | 7 | 3.3 | 2.7 |
| 5 | 0.072 | 0.27 | >100 | 1.3 | <0.5 |

^a log P from Chemdraw Ultra 11.0.1.^b % Transport in 80 min across Caco-2 cells grown for 27 days (resistance 600 ohms/cm²), measured apical to basal. Permeability measured for 4a = $4.02 \pm 0.1 \times 10^{-6}$ cm/s.

Table 4

Anti HCV activity (EC_{50} , EC_{90}) and cell cytotoxicity (CC_{50}) of ProTides of (2) with amino acid variations

| Compd | AA | Genotype 1b replicon | | Huh7 CC_{50} (μ M) |
|-------|-------|----------------------|----------------------|---------------------------|
| | | EC_{50} (μ M) | EC_{90} (μ M) | |
| 4g | L-Ala | 0.01 | 0.04 | 7 |
| 4i | D-Ala | 0.24 | 0.58 | 51 |
| 4j | L-Met | 0.06 | 0.19 | 28 |
| 4k | L-Leu | 0.07 | 0.23 | 14 |
| 4l | L-Ile | 0.86 | 2.53 | 18 |
| 4m | Val | 0.19 | 0.64 | 33 |
| 2 | — | 5.71 | 24.57 | >100 |

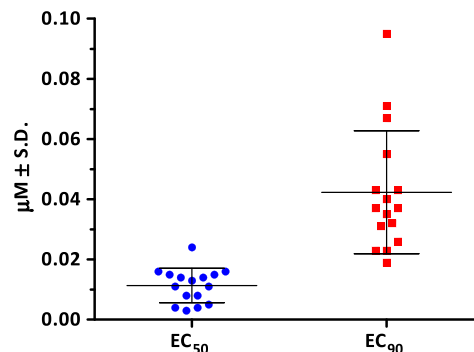
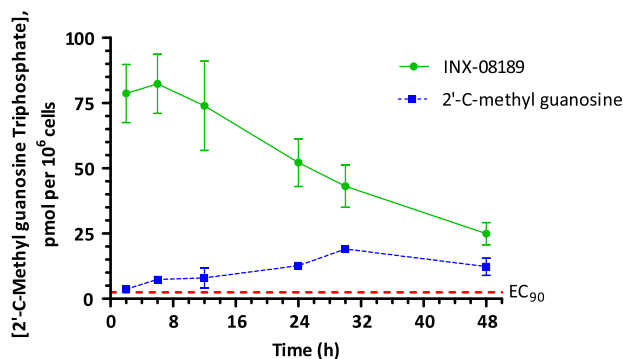


Figure 3. Potency of 4g in the HCV genotype 1b replicon assay. Data represent 16 replicates.

Figure 4. Conversion of 2 μ M Phosphoramidate 4g and 1 into 2'-C-methyl guanosine triphosphate in primary human hepatocytes. EC_{90} line is defined as the amount of intra cellular triphosphate necessary to achieve 90% inhibition in the HCV replicon assay.

highly potent inhibitor of HCV replication with EC_{50} and EC_{90} values ranging from 0.003–0.024 μ M (mean = 0.01 μ M) and 0.019–0.095 μ M (mean = 0.04 μ M), respectively. These data confirm 4g as

Table 5Data on separated diastereomers of **4g**

| Inx | Chirality | Peak | Replicon EC ₅₀ (μM) | δ _p | T _{1/2} ^a (min) |
|-------------|----------------|------|--------------------------------|----------------|-------------------------------------|
| 4g-1 | S _p | 1st | 0.044 | 4.28 (D) | 20 |
| 4g-2 | R _p | 2nd | 0.019 | 4.23 (E) | 17 |

^a Half-life in carboxypeptidase assay—see Figure 5.

the most potent nucleoside analogue based inhibitor of the HCV NS5b RNA dependent RNA polymerase reported to date. In addition, **4g** has also been tested against HCV genotypes 1a and 2 and shown to be very active, 12 and 1 nM, respectively.

To confirm that the phosphoramidate, **4g**, was being converted to the known HCV NS5b enzyme inhibitor 2'-C-methyl guanosine triphosphate, **4g** was incubated in primary human hepatocytes at an arbitrary concentration of 2 μM, and triphosphate levels were measured over a period of 48 h. In addition, the triphosphate levels

resulting from incubation with 2 μM 2'-C-methyl guanosine were also measured (Fig. 4).

The data indicates that **4g** produces a substantial C_{max} of 2'-C-methyl guanosine triphosphate of 80 pmol/10⁶ cells within the first 8 h, then decays with a half-life estimated to be over 24 h. The triphosphate of 6-O-methyl-2'-C-methyl guanosine (**2**) was synthesized (CarboSynth, Inc.), and used as an analytical standard, but none of this triphosphate was detected in multiple experiments. The nucleoside (**1**), on the other hand, slowly builds to a much delayed and lower C_{max} at 32 h compared to **4g**, consistent with its modest activity. Further work to understand the metabolism of the 6-O-methyl-2'-C-methyl guanosine phosphoramidates is underway and will be discussed in later publications. Similar to for the anti-leukaemic agent nelarabine,¹³ it appears that a 6-methoxy base group is a good pro-drug for a guanine nucleoside analogue, but here at the monophosphate metabolic level, possibly utilizing adenylate deaminase (EC 3.5.4.6) or some other cellular

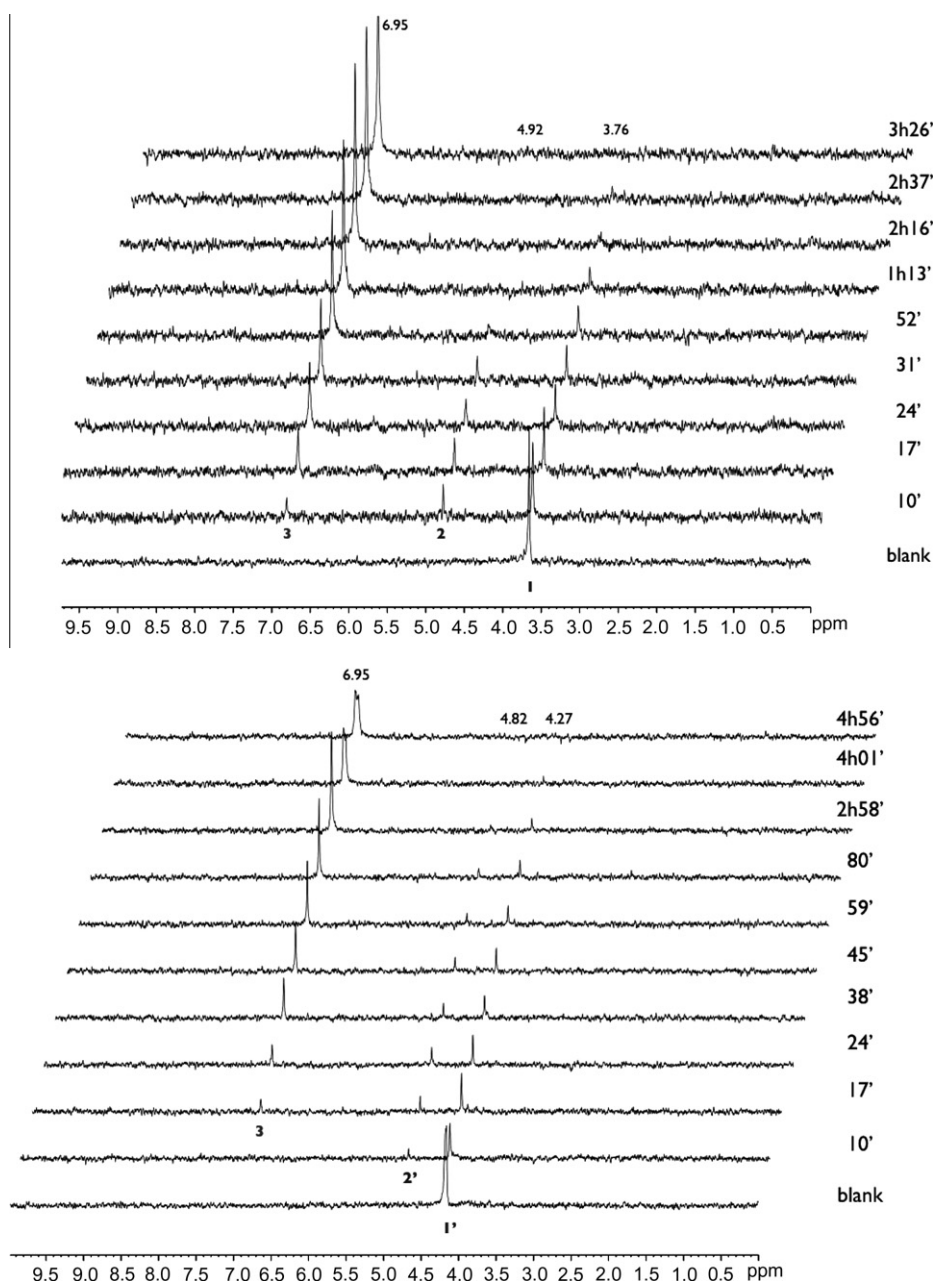


Figure 5. CPY assay on separated isomers of **4g**. Upper isomer **4g-1**, lower isomer **4g-2**. Conditions: 5 mg of compound in 200 μl of acetone + 400 μl of Trizma buffer 0.3–0.5 mg of carboxypeptidase A (purchased from SIGMA) in 200 μl of Trizma buffer.

deaminase¹⁶ These data, together with the potent HCV replicon inhibition data, demonstrate that **4g** can be metabolised rapidly in vitro to the active 2'-C-methyl guanosine triphosphate; a direct inhibitor of the HCV RNA dependent RNA polymerase NS5b.

Given the considerable interest in **4g**, the two diastereoisomers were separated on a preparative scale using a Chiral Pak AD chiral chromatography column with 1:1 ethanol/hexane as eluant (Chiral Technologies, West Chester, PA). The absolute configuration of each of the two diastereomers was determined by Vibrational Circular Dichroism (VCD) (BioTools, Inc., Jupiter, FL). Replicon data on the separate isomers are given in Table 5.

Interestingly the HCV replicon activities of the two separated diastereomers are within twofold, suggesting that the initial cleavage of the amino acid ester and subsequent elimination of the naphthol, to give the common amino acid monophosphate metabolite, happens at similar rates. A Carboxypeptidase A assay has proved a useful in vitro model of the initial metabolism of such ProTides.⁷ Thus, we carried out such an assay on the separated isomers of **4g**. The data shown in Figure 5 is a series of ³¹P NMRs taken over a 3.5 h incubation of each diastereomer (10 mM) with Carboxypeptidase A in Trizma buffer.

The ³¹P NMR traces show the parent phosphoramidate near 4.2 ppm at time zero, is rapidly cleaved by Carboxypeptidase A to the free amino acid carboxylate (peak 2/2') with a ³¹P NMR shift near 5 ppm, and then to the aminoacyl phosphate intermediate (peak 3/3') with a ³¹P NMR shift near 7.0 ppm. The formation of this intermediate is considered essential for the activity of ProTides.^{6,7} Consistent with the replicon assay data, the estimated *T*_{1/2} values of **4g-1** and **4g-2** are similar, 20 and 17 min, respectively. Further work is underway on **4g-1** and **4g-2** that may distinguish the two diastereomers, and will be reported elsewhere.

Based on the exceptional HCV antiviral activity of **4g** (INX-08189), along with other advantageous properties, such as cell permeability, ease of synthesis, and bioavailability, it was advanced into in vivo studies supporting its selection as a clinical candidate for HCV. Full details on the DMPK studies used to support the selection of **4g** will be reported elsewhere. The agent has now progressed into human clinical trials for HCV.

Acknowledgements

This study was supported by a grant from Inhibitex, Inc. to C.M. C.M. is a board member and shareholder of Inhibitex, Inc. All authors from Inhibitex, Inc. own options and are shareholders. We would like to thank CiVentiChem, RTP, NC for scaling up INX-08189 for separation of the diastereomers and Dr. Mark Gumbleton and Mr. Mathew Smith, Cardiff University for initial Caco-2 studies. We would like to thank Helen Murphy for secretarial assistance.

References and notes

- Bostan, N.; Mahmood, T. *Crit. Rev. Microbiol.* **2010**, *36*, 91.
- Hoofnagle, J. H.; Seef, L. B. *N. Engl. J. Med.* **2006**, *355*, 2444.
- Soriano, V.; Madejon, A.; Vispo, E.; Labarga, P.; Garcia-Samaniego, J.; Martin-Carbonero, L.; Sheldon, J.; Bottecchia, M.; Tuma, P.; Barreiro, P. *Expert Opin. Emerg. Drugs* **2008**, *13*, 1.
- Eldrup, A. B.; Prhavc, M.; Brooks, J.; Bhat, B.; Prakash, T. P.; Song, Q.; Bera, S.; Bhat, N.; Dande, P.; Cook, P. D.; Bennett, C. F.; Carroll, S. S.; Ball, R. G.; Bosserman, M.; Burlein, C.; Colwell, L. F.; Fay, J. F.; Flores, O. A.; Getty, K.; LaFemina, R. L.; Leone, J.; MacCoss, M.; McMasters, D. R.; Tomassini, J. E.; Langen, D. V.; Wolanski, B.; Olsen, D. B. *J. Med. Chem.* **2004**, *47*, 5284.
- Eldrup, A. B.; Allerson, C. R.; Bennett, C. F.; Bhat, S. B. B.; Bhat, N.; Bosserman, M. R.; Brooks, J.; Burlein, C.; Carroll, S. S.; Cook, P. D.; Getty, K. L.; MacCoss, M.; McMasters, D. R.; Olsen, D. B.; Prakash, T. P.; Prhavc, M.; Song, Q.; Tomassini, J. E.; Xia, J. *J. Med. Chem.* **2004**, *47*, 2283.
- Cahard, D.; McGuigan, C.; Balzarini, J. *Mini-Rev. Med. Chem.* **2004**, *4*, 371.
- McGuigan, C.; Perrone, P.; Madala, K.; Neyts, J. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 431.
- McGuigan, C.; Gilles, A.; Madala, K.; Aljarah, M.; Holl, S.; Jones, S.; Vernachio, J.; Hutchins, J.; Bryant, D.; Gorovits, E.; Ganguly, B.; Hall, A.; Kolykhalov, A.;

- Muhammad, J.; Chamberlain, S.; Henson, G. *J. Med. Chem.* **2010**, *53*, doi:10.1021/jm1003792.
- Blight, K.; Kolykhalov, A. A.; Rice, C. M. *Science* **2000**, *290*, 1972.
- Migliaccio, G.; Tomassini, J. E.; Carroll, S. S.; Tomei, L.; Altamura, S.; Bhat, B.; Bartholomew, L.; Bosserman, M. R.; Ceccacci, A.; Colwell, L. F.; Cortese, R.; De Francesco, R.; Eldrup, A. B.; Getty, K. L.; Hou, D. S.; LaFemina, R. L.; Ludmerer, S. W.; MacCoss, M.; McMasters, D. R.; Stahlhut, M. W.; Olsen, D. B.; Hazuda, D. J.; Flores, O. A. *J. Biol. Chem.* **2003**, *278*, 49164.
- Docking results were scored based on the RMSD value calculated between the GTP base present in the original model and the base of the docked compounds.
- Gupta, M.; Nair, V. *Collect. Czech. Chem. Commun.* **2006**, *71*, 769.
- Lambe, C. U.; Averett, D. R.; Paff, M. T.; Reardon, J. E.; Wilson, J. G.; Krenitsky, T. A. *Cancer Res.* **1995**, *55*, 3352.
- Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. *J. Med. Chem.* **2009**, *52*, 5520.
- Brief synthetic and analytical details for the preparation of **2** and **4g**. To a pre-cooled (0 °C) solution of 2,3,4,5-tetra-O-benzoyl-2-C-methyl-β-D-ribofuranose (10.0 g, 17.22 mmol), 2-amino-6-chloropurine (3.2 g, 18.87 mmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (7.7 ml, 51 mmol) in anhydrous acetonitrile (200 ml) trimethylsilyl triflate (12.5 ml, 68.8 mmol) was added dropwise. The reaction mixture was heated at 65 °C for 4–6 h, allowed to cool down to room temperature, poured into saturated aqueous sodium bicarbonate (300 ml), and extracted with dichloromethane (3 × 150 ml). The combined organic phase was dried over sodium sulfate and evaporated under reduced pressure. The residue was precipitated from dichloromethane and methanol. Precipitate was filtered and washed with methanol, to give the desired compound (8.5 g, 79%) as a white solid. ¹H NMR (500 MHz, CDCl₃) δ 8.13 (dd, *J* = 1.2, 8.3, 2H), 8.02–7.94 (m, 5H), 7.65–7.60 (m, 1H), 7.58–7.45 (m, 4H), 7.35 (q, *J* = 7.7, 4H), 6.65 (s, 1H), 6.40 (d, *J* = 6.7, 1H), 5.31 (s, 2H), 5.08 (dd, *J* = 4.2, 11.6, 1H), 4.79 (dd, *J* = 6.4, 11.6, 1H), 4.74 (td, *J* = 4.2, 6.5, 1H), 1.60 (s, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 166.31 (C=O), 165.38 (C=O), 165.32 (C=O), 159.13 (C2), 152.87 (C6), 152.06 (C4), 141.42 (C8), 133.77 (C-H Bn), 133.69 (C-H Bn), 133.28 (C-H Bn), 129.90 (C-H Bn), 129.82 (C-H Bn), 129.78 (C-H Bn), 129.70 (C-H Bn), 129.41 (C Bn), 128.78 (C Bn), 128.61 (C-H Bn), 128.50 (C-H Bn), 128.41 (C-H Bn), 126.00 (C5), 88.84 (C1'), 85.68 (C2'), 79.43 (C4'), 76.07 (C3'), 63.57 (C5'), 17.77 (2'-Me).
Synthesis of 2-amino-6-methoxy-9-(2-C-methyl-β-D-ribofuranosyl) purine
To a suspension of 2-amino-6-chloro-9-(2-C-methyl-2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)purine (3.0 g, 4.78 mmol) in anhydrous methanol (36 ml) at 0 °C NaOMe in methanol (5.4 ml, 25% w/w) was added. The mixture was stirred at room temperature for 24 h then quenched by addition of amberlite (H⁺). The mixture was then filtrated and solvent was removed under reduced pressure. The resultant residue was dissolved in water (50 ml) and extracted with hexane (50 ml). The organic layer was then extracted with water (50 ml), and the combined water fractions were concentrated under reduced pressure. The residue was purified by silica gel chromatography (CHCl₃/MeOH 85:15) to give the pure compound (1.125 g, 76%) as a white solid. ¹H NMR (500 MHz, MeOD) δ 8.26 (s, 1H, H₈), 5.99 (s, 1H), 4.24 (d, *J* = 9.1, 1H), 4.08 (s, 3H), 4.04 (ddd, *J* = 2.3, 5.7, 8.6, 2H), 3.87 (dd, *J* = 3.0, 12.4, 1H), 0.96 (s, 3H). ¹³C NMR (126 MHz, MeOD) δ 162.75 (C6), 161.86 (C2), 154.50 (C4), 139.35 (C8), 115.36 (C5), 93.00 (C1'), 84.15 (C4'), 80.34 (C2'), 73.57 (C3'), 61.17 (C5'), 54.25 (6-OMe), 20.35 (2'-Me). HPLC: *t*_R = 9.00 min. (solvent gradient needed 0 → 100% ACN in H₂O 30 mins). HRMS (ESI): calcd for C₁₂H₁₇N₅O₅+Na⁺ 334.1127, found 334.1125. Calcd for C₁₂H₁₇N₅O₅·0.75 H₂O: C, 44.37; H, 5.74; N, 21.56. Found: C, 44.24; H, 5.49; N, 20.83.
Synthesis of 2-amino-6-methoxy-9-(2-C-methyl-β-D-ribofuranosyl) purine 5'-O-[α-naphthyl-(2,2-dimethylpropoxy-l-alanyl)] phosphate 4g
To 2-amino-6-methoxy-9-(2-C-methyl-β-D-ribofuranosyl) purine (1.35 g, 4.34 mmol) in THF (35 ml), phosphorochloridate (10.83 mmol, 1 M solution in THF) in THF was added, followed by addition of *N*-methyl-imidazole (1.71 ml, 21.7 mmol). The mixture was stirred overnight and the solvent was removed under reduced pressure. To remove the *N*-methyl-imidazole, the phosphoramidate was dissolved in chloroform and washed 3 times with hydrochloric acid (HCl 0.5 N). The organic layer was then dried over sodium sulfate and evaporated under reduced pressure. The residue was then purified on silica gel using CHCl₃ to CHCl₃/MeOH 95:5 as an eluent, to give the pure phosphoramidate as a white solid (510 mg, 18%). ³¹P NMR (202 MHz, CDCl₃) δ 4.33, 4.29. ¹H NMR (500 MHz, MeOD) δ 8.19–8.15 (m, 1H, H₈-naph), 7.98, 7.96 (2 × s, 1H, H₈), 7.86–7.82 (m, 1H, H₅-naph), 7.68, 7.65 (2 × d, *J* = 7.0 Hz, 1H, H₄-naph), 7.53–7.44 (m, 3H, H₂, H₇, H₆-naph), 7.39, 7.37 (2 × t, *J* = 8.0 Hz, 1H, H₃-naph), 6.01, 6.00 (2 × s, 1H, H₁), 4.67–4.64 (m, 1H, H₅), 4.63–4.59 (m, 1H, H₃, H₄), 4.09–4.05 (m, 1H, H_a), 4.04 (s, 3H, 6OCH₃), 3.75, 3.72, 3.64, 3.58 (2 × AB, *J*_{AB} = 10.5 Hz, 2H, CH₂ ester), 1.33 (d, *J* = 7.5 Hz, 3H, CH₃ Ala), 0.98, 0.96 (2 × s, 3H, 2'CCH₃), 0.85, 0.84 (2 × s, 9H, 3 × CH₃ ester). ¹³C NMR (126 MHz, MeOD) δ 175.06, 174.80 (2 × d, ³*J*_{C-N-P} = 5.0 Hz, C=O ester), 162.73 (C6), 161.86 (C2), 154.55, 154.51 (C4), 148.00, 147.95 (d, ²*J*_{C-O-P} = 3.8 Hz, ipso naph), 139.36, 139.08 (CH8), 136.27, 136.25 (C10-naph), 128.86, 128.80 (CH-naph), 127.88, 127.73 (2 × d, ³*J*_{C-C-O-P} = 6.3 Hz, C9-naph), 127.48, 126.53, 126.49, 125.97, 122.81, 122.76 (CH-naph), 116.24, 116.22 (C2-naph), 116.19, 115.63 (C5), 93.34, 93.18 (C1'), 82.32, 82.16 (2 × d, ³*J*_{C-C-O-P} = 8.8 Hz, C4'), 79.99, 79.95 (C2'), 75.52, 75.37 (CH₂ ester), 74.95, 74.70 (C3'), 68.11, 67.62 (2 × d, ²*J*_{C-O-P} = 5.0 Hz, C5'), 54.28, 54.07 (6OCH₃), 51.79, 51.71 (Ca Ala), 32.26, 32.22 (C ester), 26.79, 26.71 (3 × CH₃ ester), 20.89, 20.69 (2 × d, ³*J*_{C-C-N-P} = 6.3 Hz, CH₃ Ala), 20.34, 20.35 (2'CCH₃). HPLC *R*_f = 20.95, 21.48 min.
- Faletto, M. B.; Miller, W. H.; Garvey, E. P.; St. Clair, M. H.; Daluge, S. M.; Good, S. S. *Antimicrobial Agents Chemother.* **1997**, *41*, 1099.

INX-08189, a Phosphoramidate Prodrug of 6-*O*-Methyl-2'-*C*-Methyl Guanosine, Is a Potent Inhibitor of Hepatitis C Virus Replication with Excellent Pharmacokinetic and Pharmacodynamic Properties[▽]

John H. Vernachio,^{1*} Blair Bleiman,¹ K. Dawn Bryant,¹ Stanley Chamberlain,¹ Damound Hunley,¹ Jeff Hutchins,¹ Brenda Ames,¹ Elena Gorovits,¹ Babita Ganguly,¹ Andrea Hall,¹ Alexander Kolykhalov,¹ Yule Liu,¹ Jerry Muhammad,¹ Nicholas Raja,¹ C. Robin Walters,¹ Jin Wang,¹ Karen Williams,¹ Joseph M. Patti,¹ Geoffrey Henson,¹ Karolina Madela,² Mohamed Aljarah,² Arnaud Gilles,² and Christopher McGuigan²

Inhibitex Inc., 9005 Westside Parkway, Alpharetta, Georgia 30009,¹ and Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, United Kingdom²

Received 29 September 2010/Returned for modification 4 January 2011/Accepted 19 February 2011

INX-08189 is an aryl-phosphoramidate of 6-*O*-methyl-2'-*C*-methyl guanosine. INX-08189 was highly potent in replicon assays, with a 50% effective concentration of 10 ± 6 nM against hepatitis C genotype 1b at 72 h. The inhibitory effect on viral replication was rapid, with a 50% effective concentration (EC_{50}) of 35 ± 8 nM at 24 h. An intracellular 2'-*C*-methyl guanosine triphosphate (2'-*C*-MeGTP) concentration of 2.43 ± 0.42 pmol/10⁶ cells was sufficient to achieve 90% inhibition of viral replication. *In vitro* resistance studies confirmed that the S282T mutation in the NS5b gene conferred an approximately 10-fold reduction in sensitivity to INX-08189. However, the complete inhibition of S282T mutant replicons still could be achieved with an EC_{90} of 344 ± 170 nM. Drug combination studies of INX-08189 and ribavirin indicated significant synergy in antiviral potency both in wild-type and S282T-expressing replicons. Genotype 1b replicons could be cleared after 14 days of culture when exposed to as little as 20 nM INX-08189. No evidence of mitochondrial toxicity was observed after 14 days of INX-08189 exposure in both HepG2 and CEM human cell lines. *In vivo* studies of rats and cynomolgus monkeys demonstrated that 2'-*C*-MeGTP concentrations in liver equivalent to the EC_{90} could be attained after a single oral dose of INX-08189. Rat liver 2'-*C*-MeGTP concentrations were proportional to dose, sustained for greater than 24 h, and correlated with plasma concentrations of the nucleoside metabolite 2'-*C*-methyl guanosine. The characteristics displayed by INX-08189 support its continued development as a clinical candidate for the treatment of chronic HCV infection.

Hepatitis C virus (HCV) is one of the most important causes of chronic liver disease worldwide (36). In the United States alone, an estimated 3.9 million people are infected with HCV (16), and an estimated 10,000 to 12,000 HCV-related deaths occur annually (<http://digestive.niddk.nih.gov/ddiseases/pubs/chronichepc/>).

The HCV inhibitory activity of 2'-*C*-modified nucleosides has been well studied and has been shown to specifically inhibit HCV RNA replication both in biochemical assays and in cell-based replicon assays (6). The corresponding intracellular triphosphates of these 2'-substituted nucleosides were potent, competitive inhibitors of NS5B-catalyzed reactions *in vitro*. The incorporation of the 2'-modified monophosphates onto the 3' end of the RNA strand resulted in the efficient termination of the elongation of the growing RNA chain. Accordingly, 2'-substituted nucleosides have been shown to function as nonobligate chain terminators (5). Despite the potential of 2'-*C*-modified nucleosides in the inhibition of RNA-dependent RNA polymerase (RdRp) activity, they have failed to progress as drug candidates due to one or more of the following: lack of

oral bioavailability, poor pharmacokinetic characteristics, lack of cell penetration, and inefficient intracellular conversion to the active triphosphate (6, 12, 28). For example, the triphosphate of 2'-*C*-methyl guanosine (2'-*C*-MeGTP) has been shown to be a highly potent inhibitor of RdRp activity in biochemical assays, and the nucleoside analog 2'-*C*-methyl-guanosine (2'-*C*-MeG) had high oral bioavailability in rats, but it lacked potency in cell-based subgenomic replicon assays (12, 28).

In an effort to unlock the potential of nucleoside NS5B inhibitors, we have employed a phosphoramidate prodrug approach to improve upon the characteristics of cellular uptake and intracellular activation. This approach is designed to bypass the rate-limiting initial phosphorylation step of activation by delivering the monophosphate form of the nucleoside analog to the liver, where it can be efficiently converted to the active triphosphate (24). INX-08189 is a phosphoramidate of *O*-6-methyl-2'-*C*-methyl guanosine (Fig. 1) (26). This compound was selected from a number of phosphoramidate candidates because of its significant potency in replicon assays and its ability to efficiently generate intracellular triphosphate in primary human hepatocytes (26).

The current study reports a detailed characterization of INX-08189, including a description of its potency against mul-

* Corresponding author. Mailing address: Inhibitex, Inc., 9005 Westside Parkway, Alpharetta, GA 30009. Phone: (678) 746-1100. Fax: (678) 746-1299. E-mail: jvernachio@inhibitex.com.

[▽] Published ahead of print on 28 February 2011.

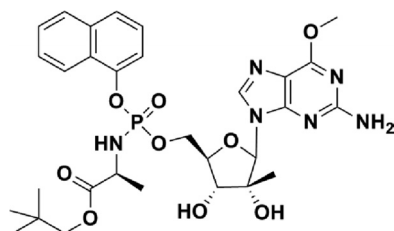


FIG. 1. Structure of INX-08189: (2*S*)-neopentyl 2-(2*R*,3*R*,4*R*)-5-(2-amino-6-methoxy-9*H*-purin-9-yl)-3,4-dihydroxy-4-methyltetrahydrofuran-2-yl(methoxy)(naphthalen-1-yloxy)(phosphorylamino)propanoate.

tiple HCV genotypes, the relationship between potency and intracellular 2'-*C*-MeGTP production, the resistance genotypes selected by the compound, and its pharmacokinetic and pharmacodynamic properties in rats and primates.

MATERIALS AND METHODS

INX-08189. INX-08189 was synthesized in the laboratory of Christopher McGuigan at the Welsh School of Pharmacy, Cardiff University, as described previously (26).

Replicon assays. The HCV inhibitory activity of INX-08189 was evaluated in replicon cell culture systems (Apath, LLC, Brooklyn, NY). For HCV genotype 1b (Con1), a Huh-7 cell line (29) expressing a stable, bicistronic subgenomic replicon encoding the Renilla luciferase reporter gene was utilized (2). For HCV genotype 2a (J6/JFH), Huh-7 cells were transfected transiently with a full-length, monocistronic HCV genome construct expressing Renilla luciferase. The inhibitory effect on HCV RNA replication using the luciferase assay and the cytotoxic effect of compounds were performed as previously described (24). For HCV genotype 1a (H77), a stable, full-length, bicistronic replicon cell line was used (3). Genotype 1a HCV RNA replication was monitored in this cell line using a quantitative reverse transcriptase PCR assay (qRT-PCR; TaqMan) as follows. Total RNA was isolated using the RNeasy 96 kit (Qiagen, Valencia, CA). The RNA preparations were quantified using the RiboGreen RNA quantitation kit (Invitrogen) and were used as the template in a qRT-PCR mixture containing 1× EZ buffer, 1× Mn acetate, 300 μM deoxynucleoside triphosphates (dNTPs), 1 μl (2.5 U) rTh, 300 nM primers specific for the HCV 3'-untranslated region (UTR) (forward, 5'-GGCTCCATCTTAGCCCTAGTC-3'; HCV-R, reverse, 5'-AGTA TCGGCACTCTCTGCAGT-3') and 150 nM TaqMan probe (6-carboxyfluorescein [FAM]-ATGCGGCTCACGGACC) (MGB probe). Amplification was performed using an ABI 7500 real-time PCR system and the following thermal cycling program: 95°C for 60 s and 60°C for 35 min, followed by 40 cycles of 95°C for 45 s and 60°C for 45 s. The HCV RNA copy number was calculated from a standard curve generated with synthetic HCV RNA standards of known concentrations.

Generation of NS5B mutant replicons. Mutations in the NS5B gene were generated in the HCV genotype 1b (Con1) subgenomic replicon. To introduce the S282T mutation in NS5B, a plasmid containing a restriction fragment incorporating the mutation (pT7GG-1b-S282T) was synthesized (Geneart Inc., Burlingame, CA), and the restriction fragment was purified and used to replace the corresponding fragment in the wild-type replicon. NS5B mutations I585T and P540T were generated by *in vitro* mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, an XhoI/SpeI fragment from the HCV genotype 1b replicon plasmid was cloned into pBluescript SKII and used as the template for mutagenesis using the primers 5'-TCTGTAGGGGTAGGCACCTATCTACTCCCAAC-3' and 5'-GTTGGGGAGTAGA TAGGTGCTACCCCTACAGA-3' for I585T and 5'-AACTCACTCCAATCA CGGCTGCGTCCCAG-3' and 5'-CTGGGACGCGAGCCGTGATTGGAGTG AGTT-3' for P540T. The XhoI/SpeI fragments containing the mutations then were cloned into the HCV genotype 1b replicon plasmid. To generate the HCV genotype 1b replicon carrying the NS5B double mutation S282T+I585T, an SfiI restriction fragment carrying the S282T mutation was used to replace the SfiI fragment in the replicon containing the I585T mutation. The replicon carrying NS5B mutations S96T and N142T was generated as follows. The S96T mutation was generated by overlap extension PCR. In the first step, two overlapping PCR fragments were amplified from the wild-type replicon using primers XhoI-F1 (5'-GAA ATT CCC TCG AGC GAT GC-3'), S96T-R1 (5'-TTA GAT CTG

GCC GtA TGT GGG GGC GT-3'), S96T-F2 (5'-ACG CCC CCA CAT aCG GCC AGA TCT AA-3'), and MfeI-R2 (5'-CAT GAT GGT GGT GTC AAT TGG T-3') (underlining indicates the introduced restriction enzyme sites and lowercase lettering indicates point mutations introduced by the oligonucleotides). In the second step, the aforementioned overlapping PCR fragments were used as the template in a PCR mixture containing primers XhoI-F1 (5'-GAA ATT CCC TCG AGC GAT GC-3') and MfeI-R2 (5'-CAT GAT GGT GGT GTC AAT TGG T-3'). The N142T mutation was generated by PCR using primers N142T-MfeI-F3 (5'-ACC AAT TGA CAC CAC CAT CAT GGC AAA AAcTGA GGT TTT CTG CG-3') and SfiI-R3 (5'-TCG ACA GGC CGC AGC GGC CTT-3'). The two PCR fragments independently carrying S96T and N142T were cloned into the genotype 1b replicon. All replicons containing altered NS5B genes were confirmed by sequencing (SeqWright, Houston, TX).

Transient transfection of NS5B mutant replicons. Replicon RNA for transfection was prepared as follows. Replicon plasmid DNA was linearized with ScaI (Fermentas, Glen Burnie, MD) and used for *in vitro* reverse transcription using the T7 MegaScript kit (Ambion, Austin, TX). The DNA template was removed by digestion with Turbo DNase, and the RNA was precipitated with 2.5 M LiCl. RNA was quantified using the Quant-iT RiboGreen RNA kit (Molecular Probes, Eugene, OR). In preparation for transfection, Huh-7 cells were cured of replicons by prolonged treatment with alpha interferon 2A (IFN-α-2A). Cured Huh-7 cells were treated with trypsin, washed three times with ice-cold phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA), and resuspended at 1.6 × 10⁷ cells/ml in PBS. Ten μg of replicon RNA was combined with 0.35 ml of cell suspension and immediately pulsed three times (800 V, 100 μs) using a BTX ElectroSquare Porator ECM 830 (Harvard Apparatus, Holliston, MA). Electroporated cells were incubated at room temperature for 10 min prior to resuspension in 20 ml of Dulbecco's modified essential medium (DMEM)-high glucose medium (HyClone, Logan, UT) supplemented with 9% fetal bovine serum (FBS) (HyClone), 2 mM glutamine (Invitrogen), and 100 U/ml PenStrep (Invitrogen). Resuspended cells were plated into 96-well BioCoat collagen-treated tissue culture plates (VWR, West Chester, PA).

HCV replicon clearance studies. HCV replicon 1b cells were seeded in 6-well plates at 1 × 10⁵ cells/well without the selective antibiotic G418. INX-08189 was added to cell cultures 4 h after seeding at the following final concentrations: 0 nM (control), 5, 10, 20, 40, and 80 nM. The medium was changed daily, and the cells were subcultured on days 5 and 10. On days 0, 5, 8, 10, 12, and 14, the cell cultures were analyzed for HCV genome-encoded Renilla luciferase expression with the Renilla luciferase assay kit (Promega, Madison, WI) using a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). On days 5, 10, and 14, a portion of the INX-08189-treated and control cell cultures were seeded into T-75 tissue culture flasks and incubated without INX-08189 but in the presence of 0.5 mg/ml of the selective antibiotic G418 (Invitrogen, Carlsbad, CA). As these secondary cultures grew, individual flasks were fixed and stained with crystal violet. For cultures where there were no visible surviving colonies, the flasks were stained after 5 weeks of G418 selection.

Measurement of intracellular 2'-*C*-methyl-GTP *in vitro*. Genotype 1b replicon cells were seeded into six-well plates at 6 × 10⁵ cells/well for 18 h before being cultured in the presence of INX-08189 for 6 h. Cell samples were harvested and extracted with 70% ethanol overnight at 4°C, and extracts were dried overnight under a stream of nitrogen. The dried extracts were reconstituted with 50 μl of 10 mM *N,N*-dimethylhexylamine, 3 mM ammonium formate in H₂O. A standard curve was generated by spiking blank cell extract samples with known concentrations of 2'-*C*-MeGTP. Spiked samples were treated identically to the test samples, and processed samples were kept at 2 to 8°C before and during analysis. Endogenous ATP and GTP levels were monitored as internal quality controls of cell viability and extraction efficiency. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was performed with an Agilent 1100 series high-performance liquid chromatograph (HPLC) equipped with an XTerra MS C₁₈, 3.5 μM, 2.1- by 50-mm column (Waters, Milford, MA) and an API 4000 mass spectrometer (Applied Biosystems, Carlsbad, CA) using negative-ion polarity. The assay was linear ($r^2 \geq 0.99$) in the concentration range of 1 to 1,000 ng per 10⁶ cells, with $\geq 90\%$ accuracy and $\leq 10\%$ coefficient of variation (CV).

Drug combination studies in genotype 1b replicon assay. Compounds were tested both as single agents and in the following combinations: INX-08189 with IFN-α-2B (ProspecBio, Rehovot, Israel) and INX-08189 with ribavirin (Rbv; Research Products, Mt. Prospect, IL). Inhibition data from the replicon cultures was analyzed for drug interactions using a three-dimensional surface model based on the Bliss independence effects definition for additivity (MacSynergy II; obtained from M. N. Prichard, K. R. Aseltine, and C. Shipman, University of Michigan) (32). As suggested by the software authors and as utilized by others, volumes of synergy or antagonism greater than 25 μM² were considered minor

but significant, volumes greater than 50 $\mu\text{M}^2\%$ were considered moderate and potentially important *in vivo*, and values greater than 100 $\mu\text{M}^2\%$ indicate strong synergy and probable importance *in vivo* (10).

Pharmacokinetic studies of rats. Rat studies were conducted at Inhibitex, Inc., in accordance with NIH guidelines and by following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Inhibitex, Inc. INX-08189 was formulated in 95% Capmul MCM (Abitec Corp., Janesville, WI)–5% Tween 80 (Sigma, St. Louis, MO) and administered by oral gavage to male Sprague-Dawley rats (Taconic Farms, Germantown, NY). Blood and tissue collections were performed as a terminal procedure. Oral doses ranged from 3 to 25 mg/kg of body weight, and sampling times were 1, 2, 4, 8, 16, 24, 72, 96, and 168 h postadministration. Time points were selected to measure 2'-C-MeGTP concentrations at both the time to maximum concentration of drug in serum (T_{max}) and at the trough (24 h). To study the relationship between liver 2'-C-MeGTP levels and plasma 2'-C-MeG concentrations, the oral dose range was expanded to 3 to 300 mg/kg, approximating human equivalent doses of 30 to 3,000 mg. Plasma samples were collected at 1, 2, 4, 8, 16, 24, 72, 96, and 168 h postadministration and correlated to trough levels of 2'-C-MeGTP in liver samples collected 24 h postadministration. Blood was collected into EDTA-containing tubes (1.6 mg EDTA/ml blood; Sarstedt, Inc., Newton, NC), and the plasma was separated by centrifugation within 30 min of collection. Liver samples were snap frozen immediately upon collection in liquid nitrogen. Plasma and liver samples were stored frozen at $\leq -80^\circ\text{C}$ prior to analysis.

Pharmacokinetic studies of cynomolgus monkeys. Primate studies were conducted at MPI Research Inc., of Mattawan, MI, in accordance with protocols approved by the IACUC of MPI Research Inc. INX-08189 was formulated in 5% (vol/vol) dimethylacetamide–20% (vol/vol) Solutol HS 15–20% (vol/vol) polyethylene glycol 400–55% (vol/vol) 50 mM sodium acetate, pH 4.0, and was administered as a single oral gavage dose. The dose level of 25 mg/kg was chosen for comparison to the equivalent dose of 50 mg/kg in rats. Liver biopsy samples (four to five samples/animal) were collected from two animals/group/time point at 3 and 8 h postdose in an effort to measure 2'-C-MeGTP levels near the T_{max} . Anesthesia was induced and maintained, and pre- and postoperative procedures were performed according to MPI Research standard operating procedures. The liver biopsy samples were pooled for each animal. Blood samples (approximately 1.2 to 2.0 ml) were collected into tubes containing sodium heparin from the femoral artery/vein or from the portal vein cannula at 0.5, 1, 2, 3, 4, 8, 12, and 24 h postadministration. Time points were selected to measure 2'-C-MeG concentrations at both the T_{max} and at the trough (24 h). Plasma was separated by centrifugation and stored frozen until analysis.

Bioanalysis of pharmacokinetic samples. The concentration of 2'-C-MeGTP in liver samples and the concentration of 2'-C-MeG in plasma samples from rats and primates was performed by LC-MS/MS as described previously (24). The assay measuring 2'-C-MeGTP in rat or primate liver was linear ($r^2 \geq 0.99$) in the concentration range of 100 to 4,000 ng per g of tissue, with $\geq 85\%$ accuracy and $\leq 2\%$ CV. The assay measuring 2'-C-MeG in rat or primate plasma was linear ($r^2 \geq 0.99$) in the concentration range of 2 to 1,250 ng/ml, with $\geq 90\%$ accuracy and $\leq 2\%$ CV. The concentrations of INX-08189 in plasma samples collected from primates were measured by LC-MS/MS as follows. Fifty μl of each test sample was added to 200 μl of acetonitrile containing an internal standard. The samples were centrifuged at $1,300 \times g$ at 4°C for 20 min, and 50 μl of supernatant from each sample was diluted with 50 μl H_2O . Samples were covered, mixed by being vortexed, and maintained at 2 to 8°C before and during analysis. Calibration curves were generated by spiking various concentrations of INX-08189 into control plasma samples. Fifteen μl of each test sample was analyzed by LC-MS/MS. Liquid chromatography was performed with an Agilent 1100 series HPLC system equipped with a Synergi 4- μm Polar-RP 30- by 2.0-mm column (Phenomenex, Torrance, CA). The HPLC system was coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectrometry was performed in positive-ion mode, and data were analyzed using Analyst v1.4.2 software (Applied Biosystems, Framingham, MA). The assay was linear ($r^2 \geq 0.99$) in the concentration range of 10 to 1,000 ng/ml, with $\geq 90\%$ accuracy and $\leq 10\%$ CV.

Pharmacokinetic analysis. Noncompartmental pharmacokinetic analyses were performed on the plasma and liver concentration data for the analytes using WinNonlin v5.2 software (Pharsight, St. Louis, MO). The extravascular dosing pharmacokinetic model was used to calculate the pharmacokinetic parameters, which included maximum observed concentration (C_{max}), time at maximal concentration (T_{max}), terminal half-life ($t_{1/2}$), and trapezoidal area determined from the plasma concentration time data from time zero to last observed concentration (AUC_{0-t}), from time 0 to 24 h (AUC_{0-24}), and from time 0 extrapolated to infinity ($\text{AUC}_{0-\infty}$). Estimates for the terminal half lives were obtained using

TABLE 1. INX-08189 potency in wild-type replicons^a

| HCV genotype | Incubation period (h) | EC ₅₀ (μM) | EC ₉₀ (μM) | CC ₅₀ (μM) for Huh-7 |
|--------------|-----------------------|------------------------------------|------------------------------------|--|
| 1b | 24 | 0.035 \pm 0.008 | 0.600 \pm 0.453 | |
| 1b | 48 | 0.011 \pm 0.005 | 0.061 \pm 0.033 | |
| 1b | 72 | 0.010 \pm 0.006 | 0.038 \pm 0.020 | 7.01 \pm 1.97 |
| 1a | 72 | 0.012 \pm 0.004 | 0.042 \pm 0.011 | 7.01 \pm 1.97 |
| 2a | 72 | 0.0009 \pm 0.0001 | 0.0065 \pm 0.001 | 7.01 \pm 1.97 |

^a All data are means \pm standard deviations from at least three independent experiments.

regression analysis. Values that were below the lower limit of quantitation were assigned the value of zero for the analyses.

Mitochondrial toxicity assay. Mitochondrial toxicity was measured by analyzing the ratio of the mitochondrial genome copy number to a nuclear gene copy number before and after drug treatment. The mitochondrial target sequence corresponded to the region between the genes TRNL1, encoding tRNA-leu, and ND1, encoding NADH dehydrogenase subunit 1, and the cellular genome comparator was the β -globin gene (7). Total DNA from untreated CEM cells was serially diluted and used to generate a standard curve for determining the absolute copy number of the gene targets. After drug treatment, total DNA was isolated from treated cells using the DNeasy tissue kit (Qiagen, Valencia, CA). The purified DNA was quantified by spectrophotometry using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). Duplex quantitative PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using a 25- μl reaction volume containing $1 \times$ QuantiTect multiplex PCR (with 6-carboxy-X-rhodamine) mix; 250 nM mitochondrion-specific primers 5'-CCACCCAAGAACAGGGTTTG-3' (forward) and 5'-TAAGAAGAGGAA TTGAACCTCTGACTG-3' (reverse); 250 nM mitochondrion-specific probe FAM-5'-TAAGATGGCAGAGCCCGGTA-3'-minor groove binding nonfluorescent quencher (MGBNFQ); 500 nM β -globin-specific primers 5'-GTGGAT GAAGTTGGTGGTGAGG-3' (forward) and 5'-CTCCACATGCCAGTTTC TATTG-3' (reverse); 250 nM globin-specific probe VIC-5'-CCTGGGCAGGT TGGTA-3'-MGBNFQ (Integrated DNA Technologies, Coralville, IA); and genomic DNA template. Amplification was carried out using the following thermal cycling program: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and then 60°C for 1 min. The ratios between mitochondrial and cellular genome copy numbers were derived for each sample and compared to those of control cultures. Changes in mitochondrial copy number were expressed as a percent difference from the control value.

RESULTS

Activity of INX-08189 against wild-type HCV replicons. The HCV inhibitory activity of INX-08189 was evaluated in replicons expressing HCV genomes from genotypes 1a, 1b, and 2a. INX-08189 was found to be a highly potent inhibitor of HCV replication, with EC₅₀s of 10 nM against genotype 1b, 12 nM against genotype 1a, and 0.9 nM against genotype 2a after 72 h of exposure (Table 1). Following 72 h of exposure, the concentration resulting in 50% cellular cytotoxicity (CC₅₀) in cultured Huh-7 cells was 7.01 μM , resulting in therapeutic indices ranging from 584 to 7,778.

To determine the magnitude of HCV inhibitory activity that could be achieved with less than 72 h of incubation, genotype 1b replicon cells were incubated with INX-08189 for 24 or 48 h, and the EC₅₀s and EC₉₀s were determined (Table 1). Similar levels of potency were observed when cells were incubated with INX-08189 for 48 or 72 h. If exposure time was reduced to only 24 h, the concentration required to achieve the EC₅₀ level increased 3.5-fold; however, INX-08189 still was able to inhibit HCV replication by 90% (EC₉₀ = 0.6 μM).

Intracellular metabolism in HCV replicon cells. The intracellular conversion of INX-08189 to 2'-C-MeGTP was deter-

TABLE 2. Intracellular 2'-C-methyl GTP in genotype 1b replicon upon incubation with INX-08189^a

| INX-08189 concn (nM) | Amt of intracellular 2'-C-methyl GTP (pmol/10 ⁶ cells) | Estimated equivalent liver concn ^b (pmol/g tissue) |
|--------------------------|---|---|
| 1× EC ₅₀ (10) | 0.84 ± 0.36 | 84 |
| 1× EC ₉₀ (40) | 2.43 ± 0.42 | 243 |
| 2× EC ₉₀ (80) | 4.97 ± 0.80 | 497 |

^a All values are means ± standard deviation from six determinations, all measured at 6 h of incubation with INX-08189.

^b Estimates are based on a liver cellularity of 1 × 10⁸ cells per g.

mined in genotype 1b replicon cells. Replicon cells were incubated with concentrations of INX-08189 that produce 50% inhibition of HCV replication (EC₅₀ = 10 nM), 90% inhibition (EC₉₀ = 40 nM), and twice the EC₉₀ (2× EC₉₀ = 80 nM). Cells were harvested after 6 h of exposure to INX-08189, and the intracellular 2'-C-MeGTP concentrations were measured (Table 2). There was a linear relationship ($R^2 = 0.91$) between the measured intracellular 2'-C-MeGTP concentrations and the concentrations of INX-08189. At 40 nM INX-08189, the concentration of 2'-C-MeGTP in Huh-7 cells was 2.43 ± 0.42 pmol/1 × 10⁶ cells. Given an estimated liver cellularity of 1 × 10⁸ cells per g (37), the intracellular concentration of 2'-C-MeGTP that would be expected to result in 90% viral inhibition in liver tissue was calculated to be 243 pmol/g of tissue. This value was useful for interpreting the relevance of liver 2'-C-MeGTP levels measured in subsequent *in vivo* pharmacokinetic studies.

To determine the intracellular half-life of 2'-C-MeGTP, actively dividing genotype 1b replicon cells were incubated with 1 μM INX-08189 for 8 h, at which time the drug was removed. Intracellular 2'-C-MeGTP levels were measured at 12, 24, 32, 48, and 56 h after the drug was initially added (Fig. 2). Based on these data, the half-life of intracellular 2'-C-MeGTP in Huh-7 cells was calculated to be approximately 24 h.

Selection and characterization of mutants resistant to INX-08189. To determine the resistance mutations selected by INX-08189, long-term cultures of genotype 1b and 1a replicons were performed in the presence of G418 and with concentrations of INX-08189 that were increased up to eight times the EC₅₀ (80 nM) in the 1b replicon and 4× the EC₅₀ (40 nM) in the 1a replicon. The establishment of stable INX-08189 escape mutants was significantly delayed, requiring selection during a 6- to 9-week span in culture. In total, five single-colony-derived resistant cell lines were selected in the genotype 1b background, and one was selected in the genotype 1a background. The nucleotide sequences of the NS5B genes from these clones were determined. Two consistent NS5B mutations, a codon 282 alteration from serine to threonine (S282T) and a change in amino acid position 585 from isoleucine to threonine (I585T), were identified in drug-resistant clones from the genotype 1b background. The S282T substitution had been identified previously as a resistance mutant for 2'-C-Me-GTP (28). In the single clone derived from the genotype 1a replicon selection, the only alteration in the NS5B gene sequence was a codon change from alanine to threonine in amino acid position 540 (A540T in genotype 1a). To determine if these amino acid substitutions were sufficient to confer resistance to INX-08189,

they were introduced singly or in combination into the genotype 1b replicon background, and potency was assessed in a transient replication assay. For comparative purposes, a replicon carrying the substitutions S96T and N142T that has been shown to confer resistance to 4'-azidocytidine (R1479) (20) was tested in parallel. INX-08189 potency against each of these mutant replicons and their relative replication competencies are summarized in Table 3. The presence of the S282T mutation resulted in drastically reduced replication efficiency, at approximately 4% of the rate of the wild-type replicon. Combining the I585T mutation with S282T improved replication efficiency to 8% of the wild-type level. INX-08189 potency against S282T mutant replicons was reduced approximately 10-fold, while INX-08189 potency against all other mutant replicons tested was unaffected, suggesting that S282T was sufficient to produce an INX-08189-resistant phenotype. Despite the shift in potency observed, exposure to INX-08189 still could significantly inhibit HCV replication in the S282T mutant replicons with an EC₉₀ of 344 ± 170 nM (Fig. 3).

Effect of combining INX-08189 and Rbv. In clinical trials, INX-08189 likely will be used in combination with standard therapy, which currently consists of pegylated interferon and Rbv. We therefore characterized the INX-08189 resistance mutant S282T as well as the other mutant replicons for their sensitivity to both Rbv and alpha interferon 2b (Table 3). Replicons expressing S282T proved to be more sensitive to the inhibitory activity of ribavirin, with an approximately 6-fold improvement in the EC₅₀, whereas the potency of IFN-α was equivalent across all mutants tested. The difference in ribavirin potency between wild-type and S282T mutants was significant ($P = 0.0093$, unpaired *t* test with Welch's correction.) To explore this effect further, drug combination studies of INX-08189 paired with Rbv were carried out in both wild-type and transiently expressed S282T replicons (Table 3). The combination of INX-08189 and Rbv was highly synergistic in the wild-type replicon, with a synergy volume of 808 μM²%. Likewise, INX-08189 combined with Rbv against replicons expressing the S282T mutation was found to be significantly synergistic (117 μM²%).

Clearance of replicons. To assess the antiviral effect of long-term treatment with INX-08189 on HCV replication, genotype

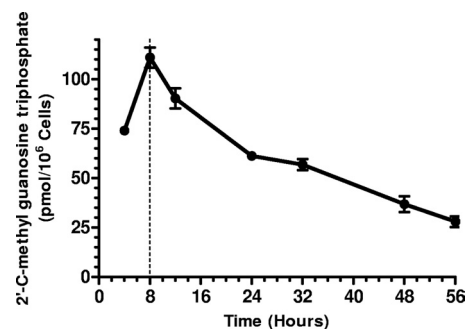


FIG. 2. Determination of 2'-C-MeGTP intracellular half-life in genotype 1b replicon. Genotype 1b replicon cells were incubated with 1 μM INX-08189 for 8 h, at which point the drug was removed. Intracellular 2'-C-MeGTP concentrations were measured by LC-MS/MS. Symbols are means ± SD for triplicate determinations in a single experiment.

TABLE 3. INX-08189 potency against transient replicons expressing mutant NS5B^a

| NS5B sequence | Replication efficiency ^c (%) | EC ₅₀ of: | | | INX-08189 + Rbv synergy ^d (μM ² %) |
|--------------------|---|----------------------|----------------|-----------------------------|--|
| | | INX-08189 (μM) | Rbv (μM) | IFN-α EC ₅₀ (IU) | |
| Con1 WT | 100 | 0.006 ± 0.003 | 12.157 ± 1.753 | 4.974 ± 1.511 | 808 |
| S282T | 4 | 0.074 ± 0.026 | 1.728 ± 0.016 | 3.116 ± 0.439 | 117 |
| I585T | 165 | 0.005 ± 0.001 | 15.247 ± 1.639 | 3.380 ± 1.383 | |
| A540T ^b | 86 | 0.004 ± 0.001 | 17.250 ± 3.235 | 3.294 ± 1.251 | |
| S282T/I585T | 8 | 0.055 ± 0.018 | 1.736 ± 0.110 | 1.937 ± 0.662 | |
| S96T/N142T | 102 | 0.006 ± 0.001 | | | |

^a All data are averages ± standard deviations from at least three independent experiments.

^b The genotype 1b strain Con1 encodes a proline at position 540, as opposed to the alanine in genotype 1a strain H77.

^c Percent luciferase activity compared to activity of the wild-type replicon.

^d Bliss independence effects definition for additivity (MacSynergy II).

1b replicon cells were cultured in the presence of 0 (control), 5, 10, 20, 40, or 80 nM INX-08189 for 14 days in the absence of G418 selection. At various time points during the culture period, cell samples were harvested and luciferase expression was determined as a measure of HCV replication activity. As summarized in Fig. 4A, luciferase activity in treated cultures decreased over time compared to that of the control, and inhibition was concentration dependent. Culturing in the presence of 10 nM INX-08189 resulted in a >2 log₁₀ reduction in HCV replication activity. Culturing in the presence of ≥20 nM (~2× EC₅₀) resulted in a >5 log₁₀ reduction in HCV replication activity after 12 days. After 14 days of culture at concentrations of ≥40 nM (1× EC₉₀), HCV replication activity was reduced to background levels. Samples of the treated cells were harvested at days 5, 10, and 14 and subcultured without INX-08189 but in the presence of G418. Any cells remaining in the cultures that retained the expression of the replicon genome would be resistant to G418 selection and after sufficient time in culture would be detected as visibly growing colonies (Fig. 4B). Cultures that were incubated in the presence of INX-08189 for only 5 days retained the expression of the replicon at all concentrations tested, although the frequency of expressing colonies diminished with increasing INX-08189 concentration. After 10 days of INX-08189 treatment, replicon-expressing cells were completely eliminated at concentrations of ≥40 nM (1× EC₉₀). After 14 days of INX-08189 treatment, replicon-expressing cells were completely elimi-

nated from the culture at concentrations of ≥20 nM (~2× EC₅₀).

Mitochondrial toxicity. Mitochondrial toxicity has been described as a manifestation of an adverse effect associated with the long-term use of certain nucleoside analogs (15). To eval-

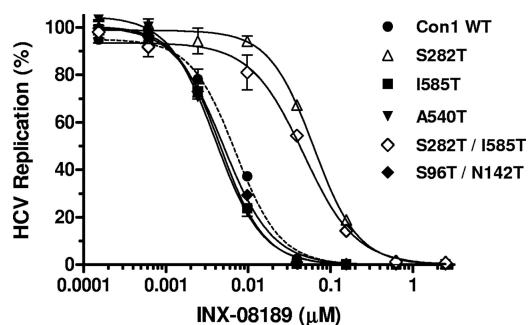


FIG. 3. Inhibition of HCV replication. Representative inhibition curves are plotted for replicons expressing wild-type (●), S282T (Δ), I585T (■), A540T (▼), S282T/I585T (◇), and S96T/N142T (◆) NS5B sequences. Concentrations of INX-08189 are indicated on the x axis, and the measured luminescent signals are expressed as a percentage of signal obtained in no-treatment controls on the y axis. Symbols are means ± SD for triplicate determinations in a single experiment.

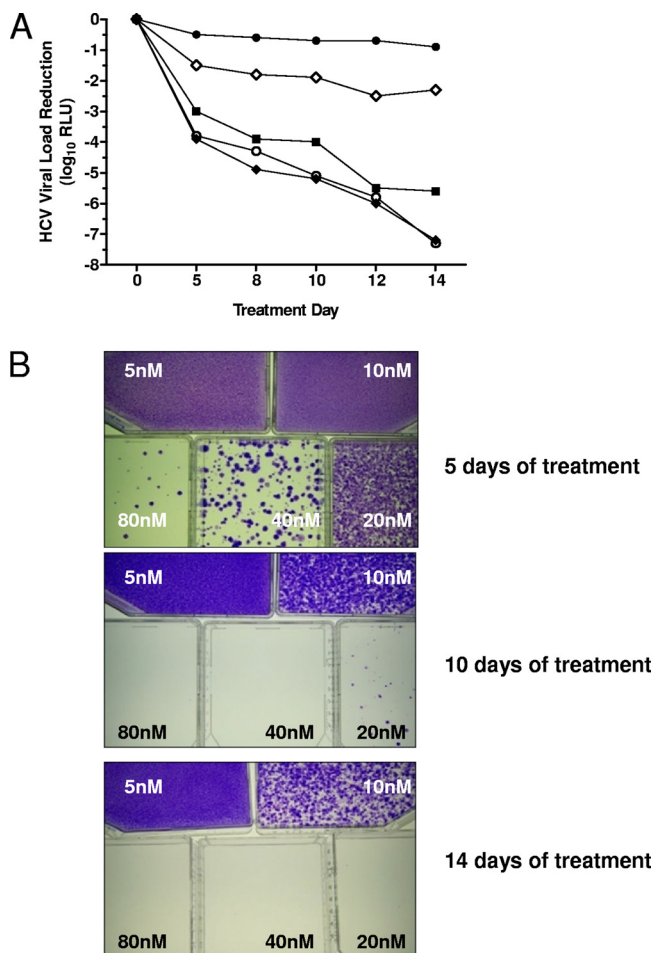


FIG. 4. Clearance of wild-type replicons *in vitro* with INX-08189. (A) Huh-7 genotype 1b replicons cells were cultured in the presence of INX-08189 without G418 for 14 days at concentrations of 5 (●), 10 (◇), 20 (■), 40 (○), and 80 nM (◆). Luciferase expression was monitored in the cultures at the indicated time points and expressed as log₁₀ change in luminescence. (B) Cells were subcultured under G418 selection for up to 5 weeks, and surviving colonies were fixed and stained with crystal violet.

TABLE 4. Mitochondrial toxicity

| Cell line | Compound | Relative mitochondrial copy no. ^a (% of control) on treatment day: | |
|--------------------|-----------|---|----------|
| | | 3 | 14 |
| CEM ^b | INX-08189 | 89 ± 11 | 102 ± 14 |
| | 2'-C-MeG | 109 ± 43 | 133 ± 4 |
| | ddC | 31 ± 5 | 1 ± 1 |
| HepG2 ^c | INX-08189 | 129 ± 22 | 115 ± 23 |
| | 2'-C-MeG | 103 ± 57 | 137 ± 2 |
| | ddC | 44 ± 16 | 20 ± 24 |

^a Measured as the ratio between mitochondrial genome copy number and cellular genome copy number compared to values for untreated control cultures. Values are averages from at least two experiments ± standard deviations.

^b Concentrations used for 3-day cultures were 20 μM INX08189, 100 μM 2'-C-MeG, and 5 μM ddC, and those for 14-day cultures were 1 μM INX08189, 100 μM 2'-C-MeG, and 0.5 μM ddC.

^c Concentrations used for 3-day cultures were 5 μM INX08189, 100 μM 2'-C-MeG, and 10 μM ddC, and those for 14-day cultures were 1 μM INX08189, 100 μM 2'-C-MeG, and 2 μM ddC.

uate the effect of INX-08189 on the relative mitochondrial genome copy number in the human cell lines CEM and HepG2, both a 3- and 14-day study were conducted. Mitochondrial copy numbers were determined after treatment with INX-08189 in CEM, a lymphocyte cell line, and HepG2, a human hepatocyte cell line. To distinguish mitochondrion-specific toxicity from general cellular cytotoxicity, increasing concentrations of INX-08189 were evaluated in the cell lines for 3 and 14 days. INX-08189 concentrations that did not affect overall cell viability were selected for the mitochondrial toxicity assessment. The maximum INX-08189 concentrations evaluated for mitochondrion-specific toxicity were 20 and 5 μM in CEM and HepG2 cells, respectively, treated for 3 days. In the 14-day experiments, the maximum INX-08189 concentration was 1 μM in both CEM and HepG2 cells. In CEM cells treated with a 20 μM concentration of INX-08189 for 3 days, the reduction in relative mitochondrial copy number was approximately 11% (Table 4). There were no effects on mitochondrial copy number observed in CEM cells at 1 μM for 14 days. The relative mitochondrial copy number in HepG2 cells was calculated to be 129 and 115% at 5 and 1 μM drug in 3- and 14-day treatments, respectively, demonstrating that INX-08189 did not alter the relative mitochondrial copy number in HepG2 cells. In contrast, a positive-control compound, dideoxycytosine (ddC), reduced the relative mitochondrial numbers by 56 and 69% in the 3-day study in CEM and HepG2 cells, respectively. Culturing CEM and HepG2 cells for 14 days with ddC resulted in mitochondrial copy number reductions of 80 and 99%, respectively (Table 4).

Pharmacokinetics in rats. The ability of INX-08189 to deliver a monophosphate form to the liver that subsequently is converted to 2'-C-Me-GTP after oral administration was tested in Sprague-Dawley rats. 2'-C-MeGTP concentrations were measured in rat liver samples harvested during a 7-day period (Fig. 5). At doses of ≥5 mg/kg, the concentrations of 2'-C-MeGTP in the liver exceeded the EC₉₀ soon after dosing and remained at or above this level for 72 h.

The measurement of 2'-C-MeGTP in liver tissue is the most direct approach for determining the bioavailability of INX-

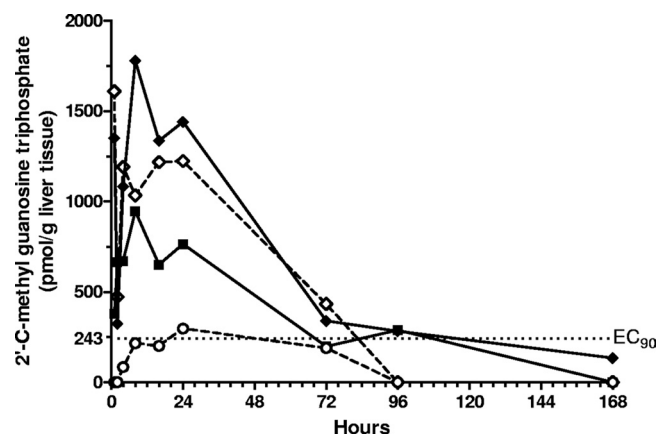


FIG. 5. Concentrations of 2'-C-methyl GTP in rat liver following oral dosing with INX-08189. Each line represents a separate dose level: 25 mg/kg (◆), 10 mg/kg (◇), 5 mg/kg (■), and 3 mg/kg (○). Symbols represent averages from two rats with the exception of the 3-mg/kg dose group, which included data from six rats. The EC₉₀ determined for Huh-7 cells (243 pmol/g) is indicated by the dotted line.

08189; however, the use of this analytical tool is limited in higher species, as it can be employed only at a few time points and is impractical for pharmacokinetic studies of humans. Another approach is to monitor the generation of a metabolite of INX-08189 in the plasma that is proportional to the production of 2'-C-MeGTP in the liver. To this end, the plasma levels of 2'-C-MeG, a major metabolite of INX-08189, were measured in the rat. The plasma AUC₀₋₂₄ (in ng · h/ml) values of 2'-C-MeG were compared to the 2'-C-MeGTP C₂₄ (ng/g) concentrations after the administration of oral doses ranging from 3 to 300 mg/kg (Table 5). At doses between 3 and 150 mg/kg, both the 2'-C-MeG plasma AUCs (ng · h/ml) and the 2'-C-MeGTP liver concentrations (ng/g) at 24 h were found to be dose proportional and linear, with R² values equal to 0.96 and 0.99, respectively. In addition, the 2'-C-MeG plasma AUCs and the 2'-C-MeGTP liver C₂₄ also were found to be highly correlative (R² = 0.97) at doses between 3 and 150 mg/kg. This correlation was not maintained at the 300-mg/kg dose. These data validate the utility of measuring 2'-C-MeG exposure in the plasma as a biomarker to monitor the liver pharmacokinetics of 2'-C-MeGTP *in vivo*.

TABLE 5. Relationship between 2'-C-MeGTP concentration in liver to 2'-C-MeG exposure in plasma following a single oral dose of INX-08189 in rats

| INX-08189 dose (mg/kg) | N | Liver 2'-C-MeGTP ^a C ₂₄ (ng/g) | Plasma 2'-C-MeG ^b AUC ₀₋₂₄ (ng · h/ml) |
|------------------------|---|--|--|
| 3 | 6 | 159 | 73.4 |
| 5 | 2 | 412 | 145.6 |
| 10 | 2 | 658 | 322.5 |
| 25 | 2 | 774 | 707.9 |
| 30 | 3 | 1,263 | 1,024.3 |
| 50 | 3 | 2,880 | 2,085.3 |
| 150 | 3 | 7,130 | 3,979.2 |
| 300 | 3 | 10,610 | 4,309.3 |

^a Mean concentrations for 2'-C-MeGTP measured at 24 h.

^b Area under the curve values covering 0 to 24 h (AUC₀₋₂₄) were calculated from average 2'-C-MeG plasma concentrations measured at six time points.

TABLE 6. Intracellular 2'-C-MeGTP concentration in liver samples from cynomolgus monkeys administered a 25-mg/kg oral dose of INX-08189

| Time (h) | 2'-C-MeGTP liver concn | | |
|----------|------------------------|------|---------------------|
| | pmol/g | ng/g | Avg \pm SD (ng/g) |
| 3 | 758 | 407 | 282 \pm 177 |
| | 292 | 157 | |
| 8 | 1,377 | 740 | 624 \pm 165 |
| | 944 | 507 | |

Pharmacokinetics in cynomolgus monkeys. To determine the concentration of 2'-C-MeGTP in the liver upon INX-08189 oral dosing, four animals were administered 25 mg/kg, and surgical liver biopsy specimens were collected under anesthesia at 3 and 8 h postdose. The mean liver 2'-C-MeGTP concentration at 3 h was 282 ng/g and increased to 624 ng/g at 8 h (Table 6). The *in vitro* EC₉₀ (130.5 ng/g) was exceeded by 2.16- and 4.78-fold at the respective sample time points.

In an effort to determine the efficiency of INX-08189 extraction by the liver, cynomolgus monkeys with surgically implanted portal vein cannulas were administered 25 mg/kg INX-08189 by oral gavage. INX-08189 concentrations were measured in plasma collected from either the portal vein or the systemic circulation (femoral vein). Following a single oral dose, INX-08189 was detected in the portal circulation within 30 min of dosing and continued to be measured for up to 4 h (Fig. 6). At this dose level, INX-08189 was not detected in the systemic circulation of this animal at any of the time points tested, whereas the metabolite 2'-C-MeG was detected in the systemic circulation beginning at 1 h postdose. These data suggest that INX-08189 is efficiently extracted from the portal circulation by the liver following oral administration, which results in the formation of the active HCV polymerase inhibitor 2'-C-MeGTP in liver tissue.

DISCUSSION

The HCV RNA-dependent RNA polymerase encoded by NS5B is an attractive target for antiviral therapy. The active site of the enzyme maintains a high degree of sequence conservation across all HCV genotypes, allowing the potential for a pangenotype inhibitor with a high barrier to resistance (22, 23). Some of the most promising and widely studied NS5B inhibitors are 2'-C-methyl nucleoside analogs (5, 6, 34). Within this family of NS5B inhibitors, the triphosphate form of 2'-C-MeG has been shown to be one of the most potent inhibitors of NS5B enzyme activity, with an 50% inhibitory concentration (IC₅₀) of 0.13 μ M (28). However, the high potency of the active 2'-C-methyl GTP at the biochemical level was not reflected in the cell-based replicon assay due to poor cell penetration and a low rate of conversion of the nucleoside to the active triphosphate (13, 28).

To overcome these limitations, we have utilized an aryloxyphosphoramidate approach to modify 2'-C-methyl guanosine analogues. This approach facilitates the direct delivery of the monophosphorylated nucleoside analogue into the cell, bypassing the first rate-limiting phosphorylation step (4). The phosphoramidate prodrug strategy has been used successfully

to improve the HCV antiviral potency of several modified nucleosides (14, 19, 25, 27). In the case of 2'-C-MeG, a striking improvement in cell-based potency over that of the parent nucleoside can be achieved (>80 fold) with phosphoramidates incorporating 1-naphthyl as the aryl-leaving group (27). These prodrugs of 2'-C-MeG also have been shown to generate high concentrations of the active 2'-C-MeG triphosphate in liver tissue following oral administration in mice (24). To further improve the performance of these compounds, alterations at the C-2 and C-6 positions of the base were investigated. It was found that phosphoramidate derivatives of 6-O-methyl-2'-C-methyl guanosine demonstrated improvements in cell-based potency, enhanced lipophilicity, cell permeability, and rapid intracellular conversion to the active triphosphate in human hepatocyte cultures (26). As a result of these studies, one compound that embodied all of these improvements, INX-08189, was selected for further characterization.

In the current study, the antiviral activity of INX-08189 was evaluated in the cell-based replicon assay against three HCV genotypes. The calculated EC₅₀s in genotype 1a, 1b, and 2a replicons were 12, 10, and 0.9 nM, respectively, making INX-08189 one of the most potent nucleoside-based NS5B inhibitors characterized to date. This magnitude of potency has been observed previously only with HCV protease inhibitors and nonnucleoside polymerase inhibitors (34). The kinetics of INX-08189 antiviral activity were observed to be rapid, with an EC₅₀ of 35 nM after only 24 h of exposure with genotype 1b replicon-containing cells. Coupled with the rapid production of 2'-C-methyl GTP observed previously with INX-08189 in human hepatocytes (26), it is clear that cell entry and the metabolic conversion of INX-08189 is enhanced compared to that of the parent nucleoside, 2'-C-MeG. To determine the concentration of intracellular triphosphate required to achieve viral inhibition, 2'-C-MeGTP was quantified in genotype 1b replicon-expressing Huh-7 cells by LC-MS/MS analysis. After 6 h of incubation, a linear relationship was observed between INX-08189 concentrations and intracellular 2'-C-MeGTP concentrations (Table 3). Given these measurements and assuming a hepatocellularity of 1×10^8 cells in 1 g of liver (37), we calculated that 84 and 243 pmol of 2'-C-MeGTP per g of liver tissue would provide 50 and 90% levels of viral inhibition, respectively. These values were used to interpret the pharma-

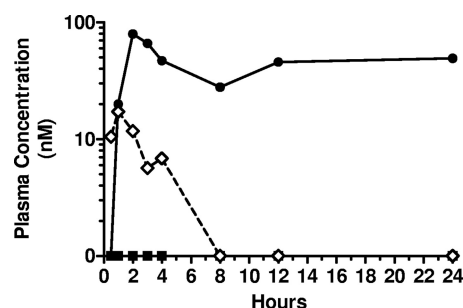


FIG. 6. Concentrations of INX-08189 and 2'-C-methyl guanosine in plasma after the oral dosing of INX-08189 in cynomolgus monkeys. Cynomolgus monkeys surgically fitted with portal vein cannulas were dosed orally with INX-08189 at 25 mg/kg. The analytes measured were 2'-C-methyl guanosine concentration in systemic plasma (●), INX-08189 in portal plasma (◊), and INX-08189 in systemic plasma (■).

codynamic relevance of the liver concentrations achieved after the oral administration of INX-08189 *in vivo* as discussed below. The intracellular half-life of 2'-C-MeGTP also was measured in the replicon cells with a value of approximately 24 h. This finding is consistent with the half-life of 2'-C-MeGTP observed in primary human hepatocyte cultures (26). The long intracellular half-life of the active triphosphate suggests that 2'-C-MeGTP concentrations in liver sufficient for antiviral activity are achievable with once-a-day dosing of INX-08189.

The ability to reduce viral loads rapidly and prevent the emergence of resistant clones during a lengthy treatment period is critical to establishing a robust sustained viral response in patients. We used the clearance of the genotype 1b replicon from Huh-7 cells as an *in vitro* surrogate to measure these characteristics of INX-08189. In the absence of G418 selection, concentrations as low as 10 nM INX-08189 ($\sim 1 \times EC_{50}$) resulted in a $\geq 2 \log_{10}$ decrease in luciferase activity, and at 40 nM INX-08189 ($1 \times EC_{90}$) a $\geq 7 \log_{10}$ decrease was observed during the 14-day culture period. Culture in the presence of G418 demonstrated that replicon-expressing cells were completely eliminated after 14 days of treatment with as little as 20 nM INX-08189 ($\sim 2 \times EC_{50}$). The ability to clear the replicon and eliminate the rebound of viral replication at such low concentrations of inhibitor are significant in light of the fact that other direct-acting antivirals, such as VX-950 (telaprevir) and HCV-796, were unable to clear the genotype 1b subgenomic replicon with up to 15 times their respective EC_{50} s (23), and PSI-7851, a phosphoramidate nucleotide of 2'-F-2'-C-methyl uridine, required >10 times its EC_{50} to clear the genotype 1b replicon at day 21 (19).

The S282T substitution in the HCV NS5B gene had been identified previously as a resistance mutant for 2'-C-methyl-modified nucleosides (20, 28, 30). To determine if INX-08189 selects for this and other mutations *in vitro*, long-term cultures of Huh-7 cells containing genotype 1a or 1b replicons were performed. As expected, the S282T mutation was observed and confirmed as a resistance mutation leading to an approximately 10-fold change in the EC_{50} . Despite this shift in potency, INX-08189 still was capable of the complete inhibition of HCV replication in the S282T mutant replicon, with an EC_{90} of 344 nM, suggesting only partial resistance to the inhibitory effect of INX-08189. Since the current standard of care for the treatment of HCV is a combination of IFN- α and Rbv, we were interested in determining what effects the S282T mutation would have on the potency of these antiviral compounds in combination. There have been previous reports of enhanced potency for Rbv against the S282T mutant both at the biochemical and cellular levels (9). Our data confirm these observations, in that we observed a 6-fold increase in potency for Rbv against HCV replicons carrying the S282T mutation. It is well documented that the S282T mutation severely limits the replication efficiency of HCV (20, 22, 28). A second mutation, I585T, was observed in the selection studies with INX-08189. As has been reported previously, the I585T mutation does not confer drug resistance; however, the presence of this amino acid change in the genotype 1b replicon improves HCV replication efficiency (35). We have shown that while compensation by I585T occurs in both wild-type and S282T-expressing replicons, it does not affect the potency of any of the treatment drugs tested. Additionally, replicon inhibition studies combin-

ing INX-08189 with Rbv demonstrated a high degree of synergy against both the wild-type and S282T mutant replicons. This is unlike some other 2'-substituted nucleosides, which are additive or antagonistic when combined with ribavirin (8). Whether the synergistic effect is due to the cooperative inhibition of RdRp enzymatic activity by metabolites of INX-08189 and Rbv or is a consequence of the downregulation of competing endogenous nucleotides by Rbv (31) remains to be determined. These data, coupled with the fact that the S282T mutation has not been detected in chronically infected HCV patients at baseline (11) or after treatment with experimental 2'-C-methyl-modified nucleoside analogues (21, 33), suggests that the combination of INX-08189 with Rbv will prove to be a highly successful strategy to minimize the generation of resistant HCV isolates clinically while at the same time optimizing viral clearance.

Mitochondrial toxicity has been observed with certain classes of nucleoside analog drugs that inhibit the activity of the mitochondrial DNA polymerase γ , and this effect has been well studied in the case of reverse transcriptase inhibitors used to treat HIV infection (1, 15). The potential liability of INX-08189 with regard to mitochondrial toxicity therefore was assessed in tissue culture studies with both a liver-derived cell line and a lymphocyte cell line. Incubation in the presence of INX-08189 for 3 or 14 days indicated no change in the ratio of mitochondrial genome copy number to cellular DNA. The results indicate a lack of mitochondrion-specific toxicity for INX-08189.

The prodrug strategy embodied by INX-08189 complicates the analysis of pharmacokinetic and pharmacodynamic properties of the molecule. In rodents, the prodrug is short lived in the plasma and cannot be measured systemically. In cynomolgus monkeys, we have shown that at a dose of 25 mg/kg, the intact prodrug was present in the portal circulation for a short time postadministration but was not detected in the systemic circulation, suggesting that at this concentration of INX-08189 the molecule was efficiently extracted by the liver. Therefore, a surrogate marker of INX-08189 bioavailability was required to study its pharmacokinetic and pharmacodynamic properties. In clinical trials using a similar prodrug strategy to deliver 2'-C-MeGTP to the liver, the nucleoside metabolite 2'-C-MeG was utilized as a surrogate analyte to monitor pharmacokinetics in HCV patients (17, 18). To validate this approach for INX-08189, we measured the generation of 2'-C-MeGTP, the active inhibitor of NS5b RdRp, which is present in the liver, and 2'-C-MeG in the plasma. In rats where both metabolites could be measured in the same animals, the data clearly indicate a linear relationship between the 2'-C-MeG AUC_{24} values and the concentration of 2'-C-MeGTP in the liver at 24 h. The data for cynomolgus monkeys, although more limited than those of the rodent studies, were consistent with this finding. The correlative relationship between the two metabolites suggests that 2'-C-MeG plasma AUC_{0-24} values can be used to assess the delivery of 2'-C-MeGTP to the liver *in vivo*. The rodent data also showed very good dose proportionality for INX-08189, demonstrating a linear relationship in the exposure of both metabolites across a wide range of doses. Based on the measurements of 2'-C-MeGTP concentrations required to achieve 90% viral inhibition *in vitro*, we evaluated if liver 2'-C-MeGTP concentrations achieved *in vivo* would be sufficient to inhibit

viral replication. In rats, the concentration of 2'-C-MeGTP in the liver 24 h postdose sufficient for 90% viral inhibition was achieved after a single oral dose of 3 mg/kg. Since this represents a trough level of 2'-C-MeGTP, multiple dosing would be expected to further improve triphosphate liver exposures. In monkeys, concentrations greater than the triphosphate EC₉₀ were measured 6 h after a single dose of 25 mg/kg. 2'-C-MeGTP in rodent liver was found to be long lived, with a half-life estimated to be greater than 24 h.

Taken together, the *in vitro* and *in vivo* data indicate that INX-08189 is a highly potent inhibitor of HCV with a high barrier for resistance and good oral pharmacokinetic properties. The data support the continued advancement of INX-08189 in clinical development for the treatment of chronic HCV infections.

ACKNOWLEDGMENTS

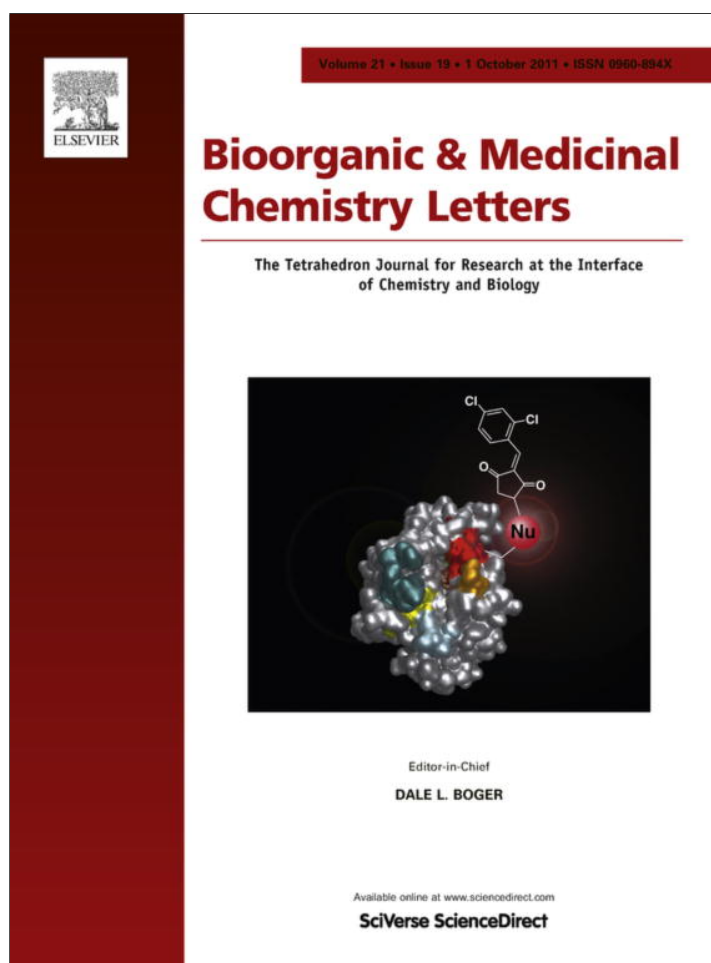
Work conducted at the University of Cardiff was supported by a grant from Inhibitex, Inc., to C.M.

C.M. is a board member and shareholder of Inhibitex, Inc.

We acknowledge Andrea Brancale, Nicola Zonta, and Sarah Jones for their assistance in modeling studies of the HCV RdRp.

REFERENCES

- Anderson, P., T. N. Kakuda, and K. A. Lichtenstein. 2004. The cellular pharmacology of nucleoside- and nucleotide-analogue reverse-transcriptase inhibitors and its relationship to clinical toxicities. *Clin. Infect. Dis.* **38**:743–753.
- Blight, K. J., A. A. Kolykhalov, and C. M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**:1972–1974.
- Blight, K. J., J. A. McKeating, J. Marcotrigiano, and C. M. Rice. 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *J. Virol.* **77**:3181–3190.
- Cahard, D., C. McGuigan, and J. Balzarini. 2004. Aryloxy phosphoramidate triesters as pro-tides. *Mini-Rev. Med. Chem.* **4**:371–381.
- Carroll, S. S., and D. B. Olsen. 2006. Nucleoside analog inhibitors of hepatitis C virus replication. *Infect. Dis. Drug Targets* **6**:17–29.
- Carroll, S. S., et al. 2003. Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. *J. Biol. Chem.* **278**:11979–11984.
- Chiu, R. W., et al. 2003. Quantitative analysis of circulating mitochondrial DNA in plasma. *Clin. Chem.* **49**:719–726.
- Coelmont, L., et al. 2006. Ribavirin antagonizes the *in vitro* anti-hepatitis C virus activity of 2'-C-methylcytidine, the active component of valopicitabine. *Antimicrob. Agents Chemother.* **50**:3444–3446.
- Cretton-Scott, E., K. Gupta, B. I. Hernandez-Santiago, and M. Larsson. 2010. Compounds and pharmaceutical compositions for the treatment of viral infections. Patent WO 2010/014134 A1.
- Delaney, W. E. T., H. Yang, M. D. Miller, C. S. Gibbs, and S. Xiong. 2004. Combinations of adefovir with nucleoside analogs produce additive antiviral effects against hepatitis B virus *in vitro*. *Antimicrob. Agents Chemother.* **48**:3702–3710.
- Dryer, P. D., et al. 2009. Screening for hepatitis C virus non-nucleotide resistance mutations in treatment-naïve women. *J. Antimicrob. Chemother.* **64**:945–948.
- Eldrup, A. B., et al. 2004. Structure-activity relationship of purine ribonucleosides for inhibition of hepatitis C virus RNA-dependent RNA polymerase. *J. Med. Chem.* **47**:2283–2295.
- Eldrup, A. B., et al. 2004. Structure-activity relationship of heterobase-modified 2'-C-methyl ribonucleosides as inhibitors of hepatitis C virus RNA replication. *J. Med. Chem.* **47**:5284–5297.
- Gardelli, C., et al. 2009. Phosphoramidate prodrugs of 2'-C-methylcytidine for therapy of hepatitis C virus infection. *J. Med. Chem.* **52**:5394–5407.
- Kakuda, T. N. 2000. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin. Ther.* **22**:685–708.
- Kim, W. R. 2002. The burden of hepatitis C in the United States. *Hepatology* **36**:S30–S34.
- Lalezari, J., et al. 2009. Antiviral activity, safety and pharmacokinetics of IDX184, a liver-targeted nucleotide HCV polymerase inhibitor, in patients with chronic hepatitis C, abstr. LB18. Abstr. 60th Annu. Meet. Am. Assoc. Study Liver Dis. Liver Meet., Boston, MA.
- Lalezari, J., et al. 2010. Antiviral activity, pharmacokinetics and safety of IDX184 in combination with pegylated interferon (pegIFN) and ribavirin (RBV) in treatment-naïve HCV genotype 1-infected subjects. *J. Hepatol.* **52**(Suppl.):S469.
- Lam, A. M., et al. 2010. PSI-7851, a pronucleotide of beta-D-2'-deoxy-2'-fluoro-2'-C-methyluridine monophosphate, is a potent and pan-genotype inhibitor of hepatitis C virus replication. *Antimicrob. Agents Chemother.* **54**:3187–3196.
- Le Pogam, S., et al. 2006. *In vitro* selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. *Virology* **351**:349–359.
- Le Pogam, S., et al. 2009. No evidence of R7128 drug resistance after up to 4 weeks treatment of GT 1, 2 and 3 hepatitis C virus infected individuals. *J. Hepatol.* **50**(Suppl.):S348.
- Ludmerer, S. W., et al. 2005. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. *Antimicrob. Agents Chemother.* **49**:2059–2069.
- McCown, M. F., et al. 2008. The hepatitis C Virus Replicon Presents a Higher Barrier to Resistance to Nucleoside Analogs than to Nonnucleoside Polymerase or Protease Inhibitors. *Antimicrob. Agents Chemother.* **52**:1604–1612.
- McGuigan, C., et al. 2010. Phosphoramidate ProTides of 2'-C-methyl-guanosine as highly potent inhibitors of hepatitis C virus. Study of their *in vitro* and *in vivo* properties. *J. Med. Chem.* **53**:4949–4957.
- McGuigan, C., et al. 2009. The application of phosphoramidate ProTide technology to the potent anti-HCV compound 4'-azidocytidine (R1479). *Bioorg. Med. Chem. Lett.* **19**:4250–4254.
- McGuigan, C., et al. 2010. Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus. *Bioorg. Med. Chem. Lett.* **20**:4850–4854.
- McGuigan, C., P. Perrone, K. Madela, and J. Neyts. 2009. The phosphoramidate ProTide approach greatly enhances the activity of beta-2'-C-methyl-guanosine against hepatitis C virus. *Bioorg. Med. Chem. Lett.* **19**:4316–4320.
- Migliaccio, G., et al. 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. *J. Biol. Chem.* **278**:49164–49170.
- Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato. 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *Cancer Res.* **42**:3858–3863.
- Olsen, D. B., et al. 2004. A 7-deaza-adenosine analog is a potent and selective inhibitor of hepatitis C virus replication with excellent pharmacokinetic properties. *Antimicrob. Agents Chemother.* **48**:3944–3953.
- Parker, W. B. 2005. Metabolism and antiviral activity of ribavirin. *Virus Res.* **107**:165–171.
- Prichard, M. N., and C. Shipman, Jr. 1990. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res.* **14**:181–205.
- Rodriguez-Torres, M., et al. 2009. Antiviral activity, pharmacokinetics, safety, and tolerability of PSI-7851, a novel nucleotide polymerase inhibitor for HCV, following single and 3 day multiple ascending oral doses in healthy volunteers and patients with chronic HCV infection, abstr. LB17. Abstr. 60th Annu. Meet. Am. Assoc. Study Liver Dis. Meet., Boston, MA.
- Schinazi, R. F., L. Bassit, and C. Gavegnano. 2010. HCV drug discovery aimed at viral eradication. *J. Viral Hepat.* **17**:77–90.
- Tomei, L., et al. 2003. Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J. Virol.* **77**:13225–13231.
- Williams, R. 2006. Global challenges in liver disease. *Hepatology* **44**:521–526.
- Wilson, Z. E., et al. 2003. Inter-individual variability in levels of human microsomal protein and hepatocellularity per gram of liver. *Br. J. Clin. Pharmacol.* **56**:433–440.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at [SciVerse ScienceDirect](http://www.sciencedirect.com)

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl



Dual pro-drugs of 2'-C-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus

Christopher McGuigan^{a,*}, Karolina Madela^a, Mohamed Aljarah^a, Arnaud Gilles^a, Srinivas K. Battina^c, Changalvala V. S. Ramamurthy^c, C. Srinivas Rao^c, John Vernachio^b, Jeff Hutchins^b, Andrea Hall^b, Alexander Kolykhalov^b, Geoffrey Henson^b, Stanley Chamberlain^b

^a Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, UK

^b Inhibitex Inc., 9005 Westside Parkway, Alpharetta, GA 30009, USA

^c CiVentiChem, 1001 Sheldon Drive, Cary, NC 27513, USA

ARTICLE INFO

Article history:

Received 10 May 2011

Revised 3 June 2011

Accepted 4 June 2011

Available online 17 August 2011

Keywords:

HCV

ProTide

Phosphoramidate

Nucleoside

Nucleotide

Polymerase

NS5B

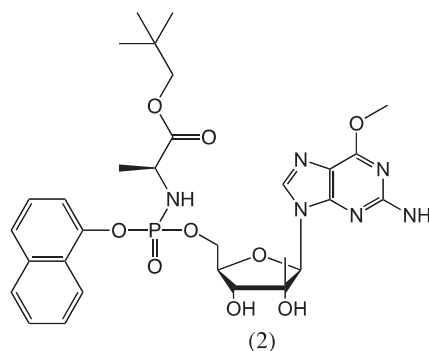
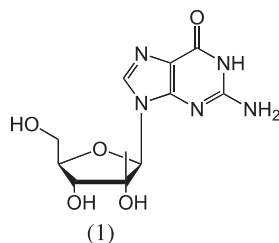
ABSTRACT

We have previously reported the power of combining a 5'-phosphoramidate ProTide, phosphate pro-drug, motif with a 6-methoxy purine pro-drug entity to generate highly potent anti-HCV agents, leading to agents in clinical trial. We herein extend this work with the disclosure that a variety of alternative 6-substituents are tolerated. Several compounds exceed the potency of the prior 6-methoxy leads, and in almost every case the ProTide is several orders of magnitude more potent than the parent nucleoside. We also demonstrate that these agents act as pro-drugs of 2'-C-methyl guanosine monophosphate. We have also reported the novel use of hepatocyte cell lysate as an *ex vivo* model for ProTide metabolism.

© 2011 Elsevier Ltd. All rights reserved.

Nucleoside analogues continue to play a vital role in the search for improved therapies for hepatitis C Virus (HCV) infection.¹ Several families of modified nucleosides have been reported as inhibitors of the HCV NS5B RNA polymerase, including 4'-modified² and 2'-modified³ nucleosides. All of these nucleosides act following sequential phosphorylation to their bioactive 5'-triphosphate

forms. As with many nucleoside analogues this phosphorylation, and particularly the first, nucleoside kinase-mediated, step may be limiting to their bio-activity.³ In these circumstances one of a number of phosphate pro-drug methods may be used.⁴ We have developed a phosphoramidate ProTide approach⁵ and applied it extensively to a variety of bioactive nucleosides. Recently we re-



* Corresponding author. Tel./fax: +44 2920874537.

E-mail addresses: mcguigan@cf.ac.uk, mcguigan@cardiff.ac.uk (C. McGuigan).

ported its effectiveness when applied to the anti-HCV agent 2'-C-methylguanosine (1).⁶ We further reported that combining the

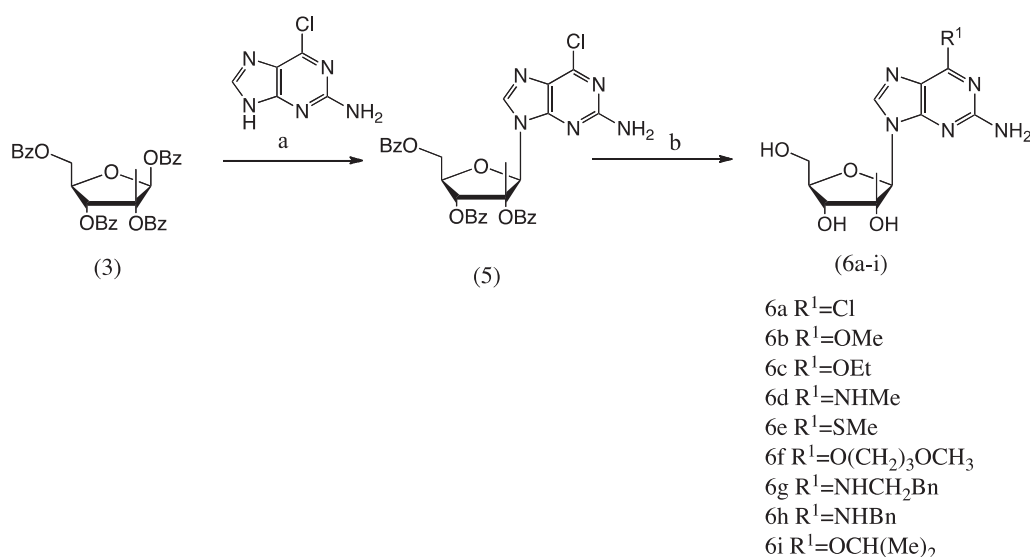


Figure 1. Reagents and conditions: (a) DBU, TMSOTf, ACN, 65 °C, 4–6 h, 76%; (b) NH_3/MeOH , 12 h, rt, 90% (for **6a**); NaOMe, MeOH, rt, 12 h, 78% (for **6b**); NaOEt/EtOH, 50 °C, 5 h, 84% (for **6c**); $\text{MeNH}_2/\text{Et}_3\text{N}/\text{EtOH}$, 85 °C, sealed tube, 92% (for **6d**); NaSMe/EtOH, rt, 18 h, 87% (for **6e**); NH_3/MeOH , rt, 12 h, 90%, NaH, 3-methoxy-1-propanol, 50% (for **6f**); BnCH_2NH_2 , EtOH, reflux, 16 h, 55%, NH_3/MeOH , rt, 94% (for **6g**); BnNH_2 , EtOH, reflux, 16 h, 98%, NH_3/MeOH , rt, 89% (for **6h**); $\text{NaOCH}(\text{Me})_2$, rt, 18 h then NaOMe, rt, 18 h, 70% (for **6i**).

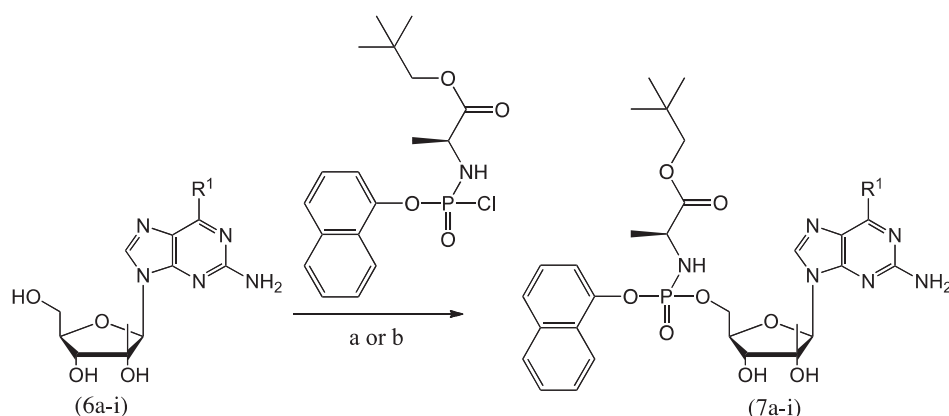


Figure 2. Reagents and conditions: (a) *N*-methylimidazole, THF, 16 h, rt, 11–28%; (b) *t*BuMgCl, THF, 22–25%.

5'-phosphate ProTide moiety with a C6 methoxy pro-drug entity further significantly boosted the potency of the compound to give the clinical candidate INX-189 (**2**) with nanomolar activity against HCV.⁷ This agent has recently successfully completed phase 1b clinical trial in HCV infected patients.

Several reasons were suggested for the potency boost presented on C6 modification. Amongst them was the ca 2-log increase in lipophilicity upon this modification, and potential consequential enhancements in passive cell uptake.⁷

Since the ProTide motif is thought to function by intracellular 5'-monophosphate release it was considered that the 5'-monophosphate from (**2**) was a substrate for adenylylase to liberate 2'-methylguanosine monophosphate as an essential precursor to the bioactive 5'-triphosphate. Given the known wide substrate specificity of adenylylase⁸ we wondered if alternative 6-substituents might substitute for the 6-methoxy and might further boost lipophilicity and enhance potency. We herein report the notable success of this approach.

As shown in Figure 1 we used the 6-chloro nucleoside as a route into various 6-substituted analogues. Thus, tetrabenzoyl 2'-C-methyl-D-ribose (**3**) was condensed with 2-amino-6-chloropurine

(**4**) in the presence of TMS triflate to give the protected beta nucleoside (**5**) in 76% yield. This was deprotected with methanolic ammonia to give (**6a**) in 78% yield. Use of alternatives deprotection/substitution conditions gave the various 6-substituted analogues (**6b–i**) as shown in Figure 1. Thus, sodium methoxide and ethoxide respectively gave (**6b–c**), methylamine (**6d**), and thiomethoxide gave (**6e**). Other analogues were similarly prepared.

The anti-HCV activity of (**6a–i**) was studied in sub-genomic replicon assay and the data are reported in Table 1. In general the compounds are active at μM levels; activity decreasing for larger C6 substituents.

Following the discovery of the power of the naphthyl neopentylalanine ProTide motif in (**2**) we now applied this moiety to (**6a–i**) as shown in Figure 2. Thus, 1-naphthyl neopentylalanine phosphoramidate was allowed to react with (**6a–i**) to generate the corresponding ProTides (**7a–i**) in moderate yield.

All compounds were isolated as roughly equimolar mixtures of phosphorus diastereoisomers as evidenced by ³¹P NMR and HPLC.⁹

The ProTides were tested as inhibitors of HCV as noted above, with data being shown in Table 2.

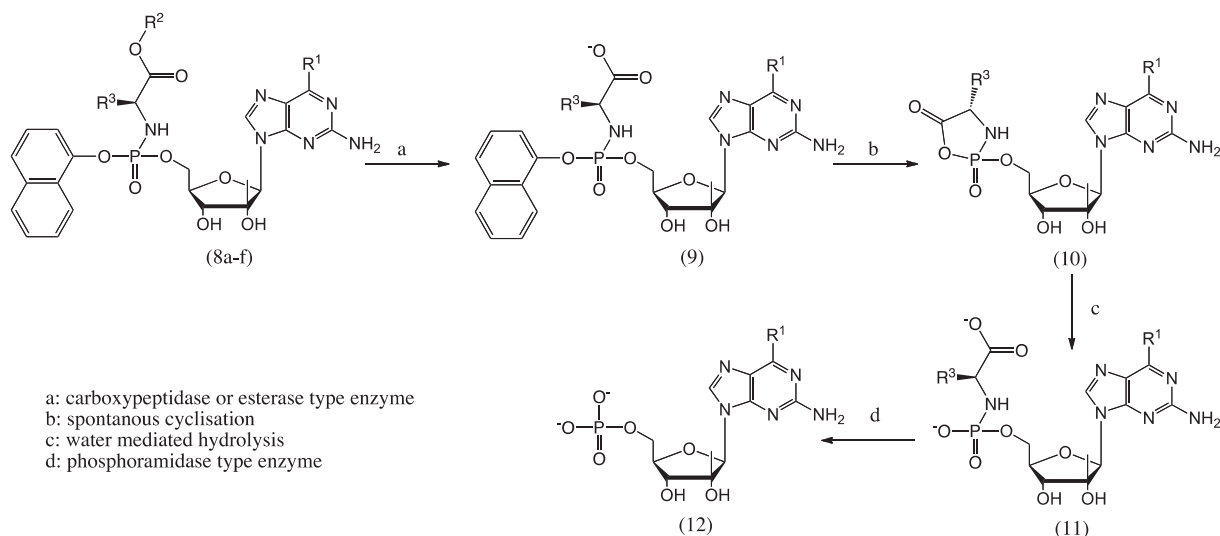


Figure 3. Putative mechanism of ProTide activation.

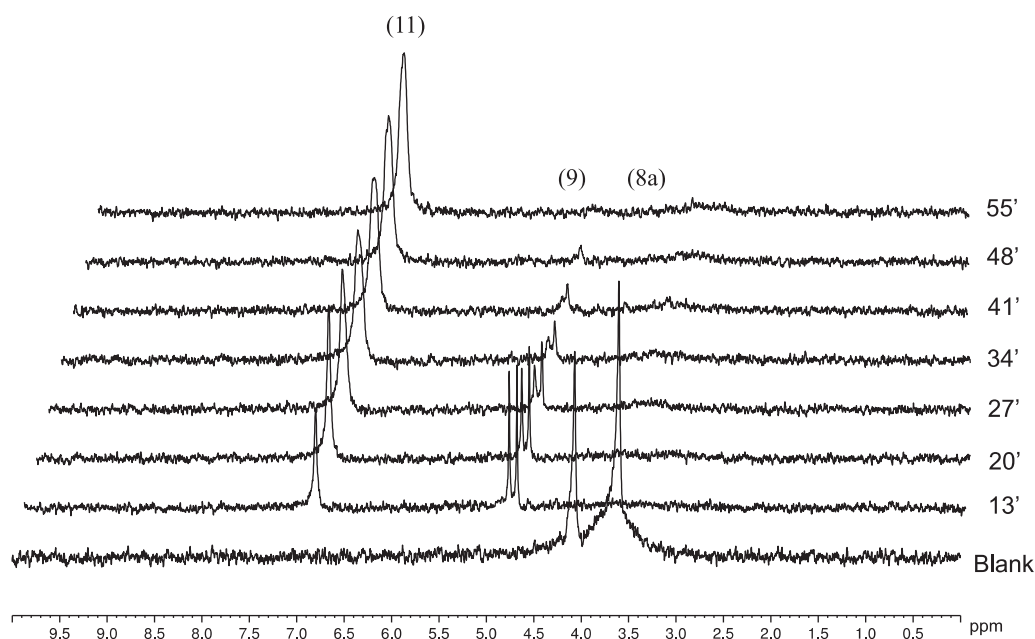


Figure 4. Carboxypeptidase Y mediated hydrolysis of (8a) followed by ^{31}P NMR.

It is striking that while all of the parent nucleosides (**6a–i**) are poorly active, with EC_{50} values of ca 5–30 μM , all of the ProTides are active sub- μM . Particularly active are the 6-ethoxy, 6-methoxy and 6-chloro compounds with EC_{90} values of <100 nM. The largest C6 substituted compounds such as (**7h**) seem to lose some activity, implying some size restrictions at C6, but even here the ProTide remains 30-fold more active than the parent (**6h**). The ProTides are cytotoxic at μM levels but their high potency still leads to therapeutic index values in the 1000 range. Given the extremely high potency of the 6-*O*-ethoxy analogue we decided to vary the ProTide motif on (**7c**). Following the same procedures as in Figure 2 but varying the phosphorochloridate we converted (**6c**) to (**8a–f**) (Table 3).

All of these compounds show sub- μM activity in replicon but none are as active as (**7c**). In general, the alanine compounds are more active than the valine ones as we have previously noted.⁶

To confirm the possible mode of action of these compounds we conducted a number of assays. In the first instance we performed a Carboxypeptidase Y assay, an in vitro probe of ProTides activation pathway (Fig. 3).

The data shown in Figures 4 and 5 represent ^{31}P NMR spectra recorded every 7 min during 14 h incubation period of (**8a**) and (**8e**) with Carboxypeptidase Y in acetone- d_6 and Trizma buffer (pH 7.6).

In case of the naphthyl benzylalanine compound (**8a**) (Fig. 4), the experiment showed fast hydrolysis of starting material ($\delta_{\text{P}} = 3.62, 4.11$ ppm) to the intermediate lacking the ester group ($\delta_{\text{P}} = 4.69, 4.83$ ppm). Both diastereoisomers of (**8a**) appear to be processed with roughly similar efficacy as far as the data allow us to discern. The single peak at 6.95 ppm corresponds to the final product of the hydrolysis—the achiral aminoacyl phosphate. The estimated half-life of (**8a**) was less than 5 min. In case

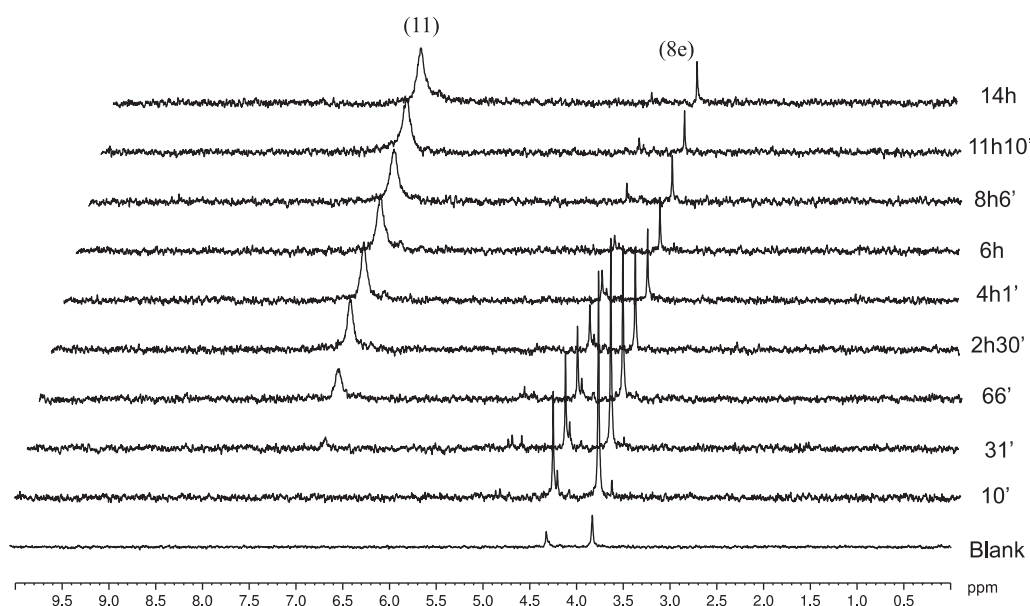


Figure 5. Carboxypeptidase Y mediated hydrolysis of (8e) followed by ^{31}P NMR.

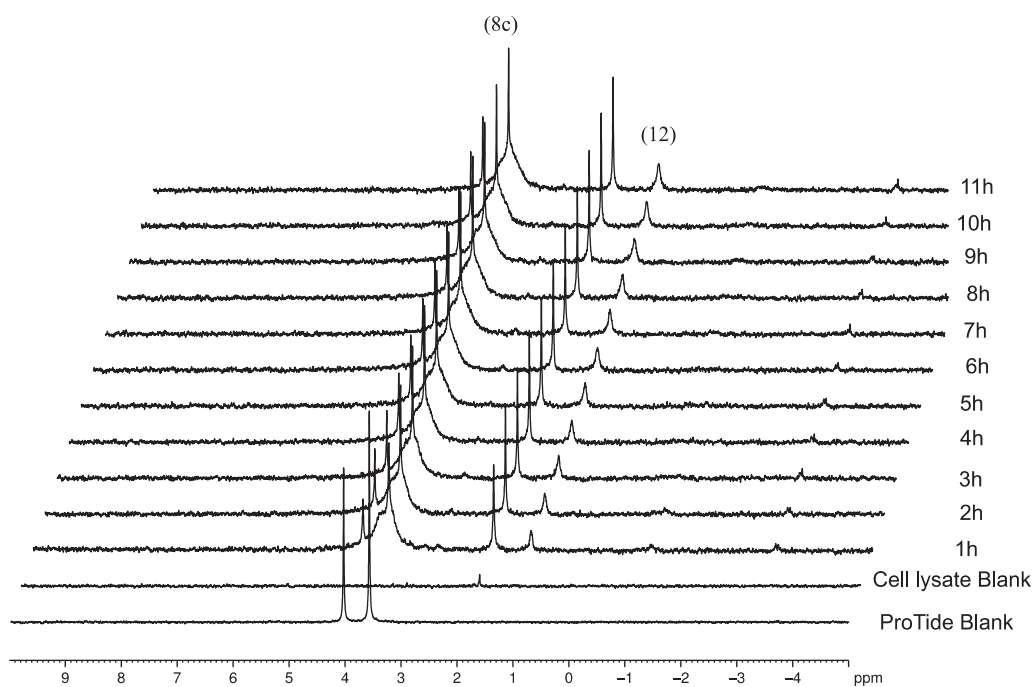


Figure 6. Metabolism of (8c) in HuH7 cell lysate followed by ^{31}P NMR.

of the isopropyl analogue (**8e**) (Fig. 5) conversion of the starting material was slower, with a half-life of 4 h and in this case one of the diastereoisomers of (**8e**) seems to be processed rather more rapidly.

These results are consistent with the replicon data, showing that (**8a**), which is processed faster, is also found to be more active ($\text{EC}_{50} = 0.04 \mu\text{M}$ vs $0.14 \mu\text{M}$ for (**8e**)). The carboxypeptidase assay is thus a potentially useful predictive model for the in vitro bio-activation of these agents.

HuH7 cell lysates have been prepared to examine monophosphate formation during the ProTide hydrolysis. An NMR based assay was performed on (**8c**) using HuH7 cell lysate (10^7 cells) in acetone- d_6 and Trizma buffer (pH 7.6) at 37°C . ^{31}P NMR spectra were recorded every 1 h for 11 h. After 1 h of incubation, the pres-

ence of newly formed peak at 0.98 ppm was observed suggesting successful liberation of the monophosphate species (Fig. 6). This signal had the same chemical shift as an authentic monophosphate under these conditions. Given that its release is considered to be a pre-requisite for antiviral action, via the triphosphate, we view these data as encouraging and consistent with the replicon data in Tables 1–3 above.

As far as we are aware, this is the first time that P-31 NMR of hepatocyte lysate has been used as an ex vivo assay of anti-HCV ProTide metabolism.

Since we regard each of these 6-modified nucleotides as pro-drugs of the guanine nucleotides, we also conducted an adenosine deaminase (ADA) assay on (**6b**), with spectra recorded each minute.¹⁰ The results are shown in Fig. 7.

Table 1
HCV Replicon activity and cytotoxicity of 6-substituted 2'-C-methylguanosine nucleosides

| Compound | R ¹ | EC ₅₀ /μM ^a | CC ₅₀ /μM ^b |
|-----------|--------------------------------------|-----------------------------------|-----------------------------------|
| 6a | Cl | 8.5 | >100 |
| 6b | OMe | 4.6 | >100 |
| 6c | OEt | 8.8 | >100 |
| 6d | NHMe | 13 | >100 |
| 6e | SMe | 11 | >100 |
| 6f | O(CH ₂) ₃ OMe | 12 | >100 |
| 6g | NHCH ₂ Bn | 24 | >100 |
| 6h | NHBn | 30 | ND |
| 6i | OCH(CH ₃) ₂ | 9.3 | >100 |

For structures see Figure 1.

^a 50% effective concentration. Replicon data for genotype 1b in HUH7 cells with 48 h exposure.

^b 50% cytotoxic concentration in HUH7 cells.

Table 2
HCV Replicon activity and cytotoxicity of 1-naphthyl neopentyl alanine ProTides of 6-substituted 2'-C-methylguanosine nucleosides

| Compound | R ¹ | EC ₅₀ ^a /μM | EC ₉₀ ^b /μM | CC ₅₀ ^c /μM |
|-----------|--------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| 7a | Cl | 0.012 | 0.028 | 16 |
| 7b | OMe | 0.010 | 0.038 | 7 |
| 7c | OEt | 0.008 | 0.032 | 10 |
| 7d | NHMe | 0.035 | 0.103 | 38 |
| 7e | SMe | 0.037 | 0.16 | 41 |
| 7f | O(CH ₂) ₃ OMe | 0.043 | 0.12 | 31 |
| 7g | NHCH ₂ Bn | 0.270 | 1.30 | 42 |
| 7h | NHBn | 0.79 | 2.7 | 15 |
| 7i | OCH(CH ₃) ₂ | 0.023 | 0.065 | 11 |

For structures see Figure 1.

^a 50% effective concentration. Replicon data for genotype 1b in HUH7 cells with 48 h exposure.

^b 90% effective concentration. Replicon data for genotype 1b in HUH7 cells with 48 h exposure.

^c 50% cytotoxic concentration in HUH7 cells.

Table 3
HCV Replicon activity and cytotoxicity of various ProTides of 6-ethoxy-2'-C-methylguanosine

| Compound | R ¹ | R ² | R ³ | EC ₅₀ /μM ^a | CC ₅₀ /μM ^b |
|-----------|----------------|----------------------|----------------|-----------------------------------|-----------------------------------|
| 8a | OEt | Bn | L-Ala | 0.04 | 11 |
| 8b | OEt | NeoPnt | L-Val | 0.17 | 24 |
| 8c | OEt | S-PhEt | L-Ala | 0.085 | 12 |
| 8d | OEt | THP | L-Ala | 0.08 | 47 |
| 8e | OEt | iPr | L-Ala | 0.14 | 40 |
| 8f | OEt | 2,4F ₂ Bn | L-Ala | 0.034 | 17 |

^a 50% effective concentration. Replicon data for genotype 1b in HUH7 cells with 48 h exposure.

^b 50% cytotoxic concentration in HUH7 cells.

These data demonstrate that (**6b**) is an excellent substrate for adenosine deaminase-mediated deamination. The final product

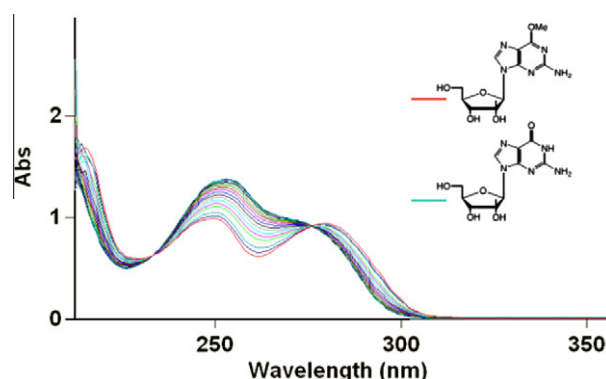


Figure 7. Adenosine deaminase mediated deamination of (**6b**) followed by UV spectroscopy.

has an identical UV spectrum to (**1**) as expected. Compound (**6c**) was found to be suitable substrate for the enzyme, however the conversion rate to (**1**) was much slower than for (**6b**). We expect that the monophosphates of (**6a–c**) released from the ProTide in vitro, may similarly be substrates for adenylate deaminase, giving the 2'-C-methylguanosine monophosphate in each case. ADA assay performed on (**6d**) and (**6e**) showed no transformation within the reaction time of 24 h which may suggest that broader substrate specificity might operate at the adenylate deaminase level or that the deamination of these nucleoside analogues and their corresponding monophosphates is catalysed by a different type of deaminase for example, human abacavir monophosphate deaminase.¹¹ To further pursue this notion we conducted the replicon anti-viral assay in the presence and absence of pentostatin, a known inhibitor of both adenosine and adenylate deaminase (data not shown) and noted that each of the ProTides herein described lost entirely their activity in the presence of pentostatin, clearly confirming an absolute need for 'deamination' to be active.

In conclusion, we herein report that a wide range of 6-substituents is acceptable at the ProTide level of 2'-C-methyl guanosine based anti-HCV agents. In every case, the parent nucleosides are poorly active, being active at only high μM levels. In every case their ProTides are active sub-μM; in most cases the potency of the nucleoside is enhanced >100 fold. Alanine emerges again as the preferred amino acid, and several ester variants lead to very potent compounds. In some cases the potency and selectivity in vitro exceeds that of the clinical candidate (**2**), INX-08189. In every case 'deamination' at the C6-position, presumably at the monophosphate level, is implicated as essential for activity, and thus these compounds are dual pro-drugs. We have also reported the novel use of hepatocyte cell lysate as an ex vivo model for ProTide metabolism.

Acknowledgements

This study was supported by a grant from Inhibitex, Inc. to C.M. C.M. is a board member and shareholder of Inhibitex, Inc. All authors from Inhibitex, Inc. own options and are shareholders. We would like to thank Helen Murphy for secretarial assistance.

References and notes

- Brown, N. A. *Expert Opin. Invest Drugs* **2009**, *18*, 709.
- Klumpp, K.; Leveque, V.; Le Pogam, S.; Ma, H.; Jiang, W.-R.; Kang, H.; Granycome, C.; Singer, M.; Laxton, C.; Hang, J. Q.; Sarma, K.; Smith, D. B.; Heindl, D.; Hobbs, C. J.; Merrett, J. H.; Symons, J.; Cammack, N.; Martin, J. A.; Devos, R.; Najera, I. *J. Biol. Chem.* **2006**, *281*, 3793.
- Eldrup, A. B.; Prhavc, M. I.; Brooks, J.; Bhat, B.; Prakash, T. P.; Song, Q.; Bera, S.; Bhat, N.; Dande, P.; Cook, P. D.; Bennett, C. F.; Carroll, S. S.; Ball, R. G.; Bosserman,

- M.; Burlein, C.; Colwell, L. F.; Fay, J. F.; Flores, O. A.; Gerry, K.; LaFemina, R. L.; Leone, J.; MacCoss, M.; McMasters, D. R.; Tomassini, J. E.; Langen, D. V.; Wolanski, B.; Olsen, D. B. *J. Med. Chem.* **2004**, *47*, 5284.
4. Gundmundsson, K. S.; Wang, Z. C.; Daluge, S. M.; Johnson, L. C.; Hazen, R.; Condreay, L. D.; McGuigan, C. *Nucleos Nucleotide Nucl.* **2004**, *23*, 1929.
 5. McGuigan, C.; Cahard, D.; Sheeka, H. M.; DeClercq, E.; Balzarini, J. *J. Med. Chem.* **1996**, *39*, 1748.
 6. McGuigan, C.; Gilles, A.; Madela, K.; Aljarah, M.; Holl, S.; Jones, S.; Vernachio, J.; Hutchins, J.; Ames, B.; Bryant, D.; Gorovits, E.; Ganguly, B.; Hunley, D.; Hall, A.; Kolykhalov, A.; Liu, Y.; Muhammad, J.; Raja, N.; Walters, R.; Wang, J.; Chamberlain, S.; Henson, G. *J. Med. Chem.* **2010**, *53*, 4949.
 7. McGuigan, C.; Madela, K.; Aljarah, M.; Gilles, A.; Brancale, A.; Zonta, N.; Chamberlain, S.; Vernachio, J.; Hutchins, J.; Hall, A.; Ames, B.; Gorovits, E.; Ganguly, B.; Kolykhalov, A.; Wang, J.; Muhammad, J.; Patti, J. M.; Henson, G. *BMCL* **2010**, *20*, 4850.
 8. Simon, L. N.; Bauer, R. J.; Tolman, R. L.; Robins, R. K. *Biochemistry* **1970**, *9*, 573.
 9. *Procedure for 7c.* To nucleoside (1 mol equiv) in anhydrous THF, phosphorochloridate (3 mol equiv) in THF was added, followed by addition of *N*-methyl-imidazole (5 mol equiv). The mixture was stirred overnight under argon atmosphere. Solvent was removed under reduce pressure. To remove the *N*-methyl-imidazole, the phosphoramidate was dissolved in chloroform and washed 3 times with hydrochloric acid (HCl 0.5 N). The organic layer was then dried over sodium sulfate and evaporated under reduce pressure. The residue was then purified on silica gel using CHCl₃ to CHCl₃/MeOH 95:5 as an eluent, to give the pure phosphoramidate as a white solid. Starting from 500 mg of nucleoside. Yield 28% (285 mg).
- ¹H NMR (500 MHz, MeOD-*d*₄) δ 8.21–8.14 (m, 1H, H-8 napht), 7.98 and 7.96 (2s, 1H, H-8), 7.86–7.79 (m, 1H, H-5 napht), 7.66 and 7.63 (2d, *J* = 8.3 Hz, 1H, H-4 napht), 7.56–7.43 (m, 3H, H-7, H-6, H-3 napht), 7.41–7.34 (m, 1H, H-2 napht), 6.03 and 6.02 (2s, 1H, H-1'), 4.67–4.71 (m, 2H, H-5', H-5''), 4.54–4.47 (2q, *J* = 7.0 Hz, 2H, OCH₂CH₃), 4.37–4.25 (m, 2H, H-3', H-4'), 4.12–4.04 (m, 1H, CH Ala), 3.69 and 3.65 (2 AB systems, *J* = 10.5 Hz, 2H, CH₂ ester), 1.41 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.33 (d, *J* = 7.1 Hz, 3H, CH₃ Ala), 0.98 and 0.96 (2s, 3H, CH₃), 0.84 and 0.82 (2s, 9H, 3 \times CH₃ ester)
- ¹³C NMR (126 MHz, MeOD-*d*₄) δ 175.06, 174.80 (2d, ³*J*_{C-C-N-P} = 5.0 Hz, C=O), 162.39 (C-6), 161.87, 161.86 (C-2), 154.57, 154.53 (C-4), 148.01, 147.96 (2d, ²*J*_{C-O-P} = 3.6 Hz, C-1 napht), 139.32, 139.04 (C-8), 136.29, 136.26 (C-10 napht), 128.84, 128.79 (C-5 napht), 127.91, 127.86 (C-6 napht), 127.75, 127.72 (C-7 napht), 126.53, 126.40 (C-9 napht), 125.96, 125.93 (C-3 napht), 122.81, 122.76 (C-4 napht), 116.23, 116.18 (2d, ³*J*_{C-C-O-P} = 2.9 Hz, C-2 napht), 115.63, 115.62 (C-5), 93.34, 93.20 (C-1'), 82.33, 82.16 (2d, ³*J*_{C-C-O-P} = 8.0 Hz, C-4'), 79.98, 79.94 (CH₂ ester), 75.37 (C-2'), 74.96, 74.72 (C-3'), 68.14, 67.67 (2d, ²*J*_{C-O-P} = 5.3 Hz, C-5'), 63.59, 63.57 (OCH₂CH₃), 51.79, 51.72 (CH Ala), 32.26, 32.23 (C(CH₃)₃ ester), 26.72, 26.69 (C(CH₃)₃ ester), 20.84, 20.64 (2d, ³*J*_{C-C-N-P} = 6.6 Hz, CH₃ Ala), 20.34, 20.31 (2'-CH₃), 14.89 (OCH₂CH₃) ³¹P NMR (202 MHz, MeOD-*d*₄) δ 4.28 and 4.21. HPLC *t*_R = 22.92, 23.16 min (Varian Polaris C18-A (10 μ M) analytic column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient 90/10 to 0/100 v/v in 30 min).
10. 1 ml of 44 μ M solution of nucleoside in phosphate buffer (pH7.4), 30 μ L of ADA solution. Spectra recorded in 1 min intervals for 30 min.
 11. Schinkmanova, M.; Votruba, I.; Shibata, R.; Han, B.; Liu, X.; Cihlar, T.; Holy, A. *Collect. Czech. Chem. Commun.* **2008**, *2*, 275–291.

Phosphorodiamidates as a Promising New Phosphate Prodrug Motif for Antiviral Drug Discovery: Application to Anti-HCV Agents

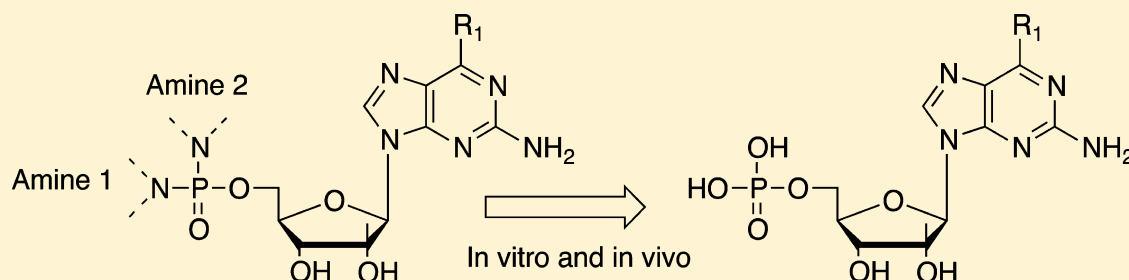
Christopher McGuigan,^{*,†} Karolina Madela,[†] Mohamed Aljarah,[†] Claire Bourdin,[†] Maria Arrica,[†] Emma Barrett,[†] Sarah Jones,[†] Alexander Kolykhalov,[‡] Blair Bleiman,[‡] K. Dawn Bryant,[‡] Babita Ganguly,[‡] Elena Gorovits,[‡] Geoffrey Henson,[‡] Damound Hunley,[‡] Jeff Hutchins,[‡] Jerry Muhammad,[‡] Aleksandr Obikhod,[‡] Joseph Patti,[‡] C. Robin Walters,[‡] Jin Wang,[‡] John Vernachio,[‡] Chandalvala V. S. Ramamurty,[§] Srinivas K. Battina,[§] and Stanley Chamberlain[‡]

[†]Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, U.K.

[‡]Inhibitex Inc., 9005 Westside Parkway, Alpharetta, Georgia 30004, United States

[§]CiVentiChem, 1001 Sheldon Drive, Cary, North Carolina 27513, United States

S Supporting Information



ABSTRACT: We herein report phosphorodiamidates as a significant new phosphate prodrug motif. Sixty-seven phosphorodiamidates are reported of two 6-*O*-alkyl 2'-*C*-methyl guanosines, with significant variation in the diamidate structure. Both symmetrical and asymmetric phosphorodiamidates are reported, derived from various esterified amino acids, both *D* and *L*, and also from various simple amines. All of the compounds were evaluated versus hepatitis C virus in replicon assay, and nanomolar activity levels were observed. Many compounds were noncytotoxic at 100 μ M, leading to high antiviral selectivities. The agents are stable in acidic, neutral, and moderately basic media and in selected biological media but show efficient processing by carboxypeptidases and efficiently yield the free nucleoside monophosphate in cells. On the basis of *in vitro* data, eight leads were selected for additional *in vivo* evaluation, with the intent of selecting one candidate for progression toward clinical studies. This phosphorodiamidate prodrug method may have broad application outside of HCV and antivirals as it offers many of the advantages of phosphoramidate ProTides but without the chirality issues present in most cases.

INTRODUCTION

There is an urgent ongoing need for improved therapeutic agents for hepatitis C Virus (HCV) infection, with an increasing emphasis on direct acting antivirals (DAAs), and in particular, inhibitors of the viral NSSB RNA polymerase.¹ Nucleoside-based inhibitors of the polymerase are considered particularly valuable on the basis of the high genetic barrier to resistance.² Thus, a number of nucleoside modifications (1–4, Figure 1) have emerged with anti-HCV activity *in vitro*, and several have progressed to clinical evaluation.

One issue common to all nucleoside analogues, either antiviral or anticancer, is an absolute need for nucleoside kinase-mediated activation to their 5'-monophosphate forms. In some cases, as in the present anti-HCV field, further phosphorylation to the 5'-triphosphate is also required, and in general the first phosphorylation step is considered rate-limiting.³ With this in mind, and given that the free phosphate derivatives are not considered useful drug entities due to poor

cell uptake, a number of phosphate (nucleotide) prodrug motifs have emerged.⁴ These have included our aryl phosphoramidate ("ProTide") approach,⁵ the amidate diester method of Wagner⁶ and McKenna,⁷ the lipid diester approach of Hosteller,⁸ thioester approaches of Gosselin,⁹ cytochrome based methods,¹⁰ and the chemical driven cycloSAL method of Meier.¹¹ Each of these methods has its strengths and weaknesses. In general, fully blocked prodrugs (ProTides, CycloSAL, etc.) may give better delivery but do often generate a chiral phosphate center leading to isomer issues. Phosphate diester methods avoid the chirality issue but may have delivery challenges.⁴ Despite this, several phosphate prodrugs of antiviral nucleosides have progressed into clinical trials. This includes Inhibitex's INX-189¹² (5, Figure 2) and Pharmasset's PSI-7977¹³ (6), both based directly on phosphoramidate ProTides, and Idenix's

Received: September 2, 2011

Published: October 31, 2011

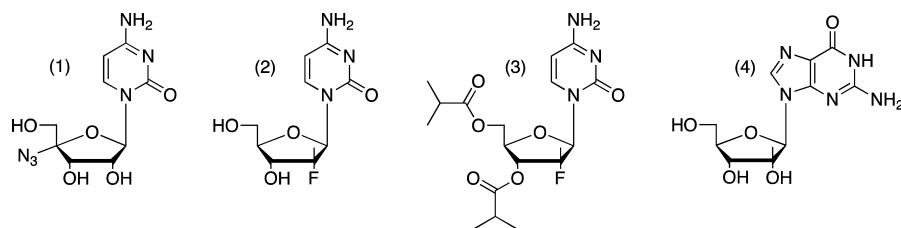


Figure 1. Some anti-HCV nucleoside analogues.

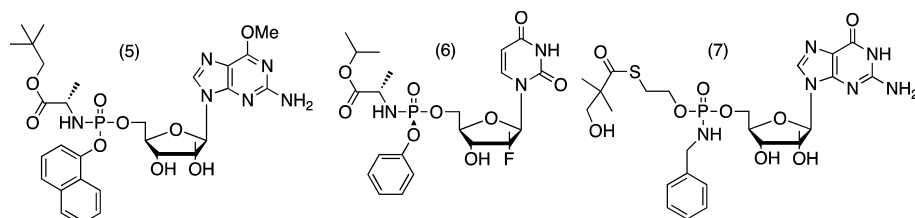
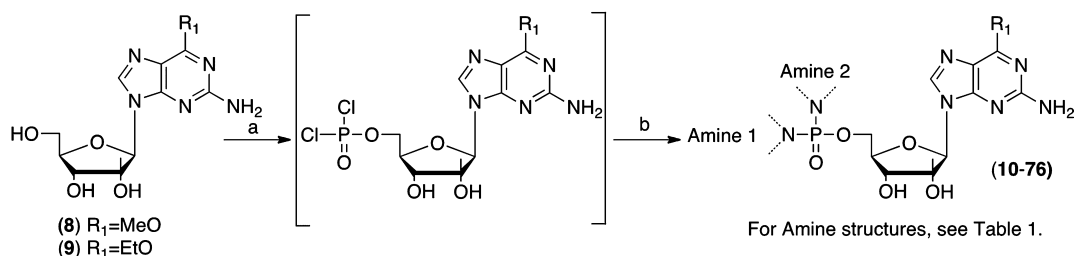


Figure 2. Some anti-HCV phosphate prodrug compounds in clinical trials.

Scheme 1. Synthetic Route to Phosphorodiamidates^a



^aReagents and conditions: (a) Et_3N (1 equiv), POCl_3 (1 equiv), dry THF; (b) for symmetrical diamidates, amine (5 equiv), Et_3N (10 equiv), dry DCM; for asymmetric diamidates, amine 1 (1 equiv), Et_3N (2 equiv) then amine 2 (5 equiv), Et_3N (10 equiv), dry DCM.

IDX184¹⁴ (7), which is a hybrid phosphoramidate/SATE prodrug. Each of these is now in human trials for efficacy versus HCV.

Most chiral nucleotide prodrugs that have entered into the clinic to date have been progressed as diastereoisomeric mixtures at the phosphate center. NewBiotics' anticancer agent NB1011 is a further example in this category.¹⁵ In general, when tested, both stereoisomers often display similar biological activity in vitro and always release the same pharmacophore after initial metabolism.¹⁶ One exception to this in the HCV field to date is Pharmasset, who has developed a large scale separation technique for their mixed compound PSI-7851, now pursued as the single stereoisomer 6 as above.¹³

With this in mind, we sought to revisit the notion of an achiral phosphate prodrug motif with a phosphoramidate core. In addition, we particularly wanted to formulate a prodrug whose promoieties were nontoxic and preferably natural. Thus our attention turned to phosphorodiamidates. Indeed, over 20 years ago, our group was among the first to report phosphorodiamidate-based nucleotide prodrugs. Thus, seeking to deliver the 5'-monophosphate of the anti-HIV agent AZT into cells, we prepared a series of L-amino acid phosphorodiamidates with a variety of esters.¹⁷ There have since been reports of this motif on phosphonates¹⁸ and non-nucleosides¹⁹ but little further work on nucleosides.^{20–22}

In this paper, we report the very promising profile of a family of such phosphorodiamidates of 2'-C-methylguanosine and its derivatives, leading toward selection of a clinical candidate.

CHEMISTRY

Following the success of 5 in early clinical trials for HCV²³ and our recent disclosure that alternative 6-alkoxy (and other) groups may be equally effective,²⁴ we used the 6-alkoxy derivatives of 2'-C-methylguanosine as our starting point. These were prepared by methods we have described.¹² Following our much earlier reports on phosphoramidates of AZT,¹⁷ we used a single synthetic route based on treating the unprotected nucleoside with phosphoryl chloride to generate the intermediate dichloridate which was not isolated (Scheme 1). In the first instance, we used the 6-methoxy analogue 8, the base nucleoside behind 5. Reaction with POCl_3 in THF in the presence of Et_3N for 45 min (when ^{31}P NMR showed no presence of POCl_3) followed by addition of an excess of various amino acid esters (or other amines), lead to compounds 10–76 as shown in Table 1.

As L-alanine is often a preferred motif in phosphoramidate ProTides and the final putative activating step for these diamidates would be the same loss of amino acid, we thought that L-alanine may be beneficial here, so our first SAR family was symmetrical substituted L-alanines with linear esters methyl-(10), ethyl-(11), *n*-propyl-(12), *n*-butyl-(13), and *n*-pentyl-(14); branched esters isopropyl-(15) and (*R,S*)-2-butyl-(16), and 3,3-dimethylbutyl-(17); followed by cyclic esters cyclobutyl-(18), cyclopentyl-(19) cyclohexyl-(20), and 2-tetrahydropyranyl-(24). We have often reported that benzyl esters can be rather effective in ProTides, so we prepared the benzyl-(21), *S*-phenethyl-(22), 2,4-difluorobenzyl-(23), and 2-indanyl-(25) L-alanine esters. Given the extremely high potency

Table 1. Replicon Data and Cytotoxicity of Nucleoside Analogues and Phosphorodiamidates

| compd | AA ester/amine | AA ester/amine | nucleoside | EC ₅₀ (μM) | | EC ₉₀ (μM) | | CC ₅₀ (μM) |
|-------|----------------------------------|----------------------------------|------------|-----------------------|-------|-----------------------|-------|-----------------------|
| | | | | av | SD | av | SD | |
| 4 | | | 2'CMeG | 2.2 | 1.4 | 8.3 | 7.5 | >100 |
| 8 | | | 6OMe2'CMeG | 4.4 | 2.2 | 19.4 | 10.2 | >100 |
| 9 | | | 6OEt2'CMeG | 9.5 | 1.84 | 24.5 | 1.78 | >100 |
| 5 | L-Ala OCH ₂ tBu | NaphO | 6OMe2'CMeG | 0.01 | 0.01 | 0.04 | 0.02 | 7.01 |
| 10 | L-Ala OMe | L-Ala OMe | 6OMe2'CMeG | 5.90 | 0.76 | >10 | | >100 |
| 11 | L-Ala OEt | L-Ala OEt | 6OMe2'CMeG | 1.20 | 0.08 | 4.10 | 0.09 | >100 |
| 12 | L-Ala OnPr | L-Ala OnPr | 6OMe2'CMeG | 0.28 | 0.05 | 1.20 | 0.53 | >100 |
| 13 | L-Ala OButyl | L-Ala OButyl | 6OMe2'CMeG | 0.07 | 0.01 | 0.23 | 0.02 | >100 |
| 14 | L-Ala OPentyl | L-Ala OPentyl | 6OMe2'CMeG | 0.03 | 0.01 | 0.11 | 0.02 | 65 |
| 15 | L-Ala OiPr | L-Ala OiPr | 6OMe2'CMeG | 0.49 | 0.18 | 2.10 | 0.09 | >100 |
| 16 | L-Ala O-(R _S)-2Bu | L-Ala O-(R _S)-2Bu | 6OMe2'CMeG | 0.15 | 0.00 | 0.53 | 0.10 | >100 |
| 17 | L-Ala O-3,3-dimethylbutyl | L-Ala O-3,3-dimethylbutyl | 6OMe2'CMeG | 0.02 | 0.01 | 0.09 | 0.07 | 71 |
| 18 | L-Ala OcBu | L-Ala OcBu | 6OMe2'CMeG | 0.32 | 0.05 | 0.80 | | >100 |
| 19 | L-Ala OcPentyl | L-Ala OcPentyl | 6OMe2'CMeG | 0.06 | 0.01 | 0.22 | 0.03 | >100 |
| 20 | L-Ala OcHx | L-Ala OcHx | 6OMe2'CMeG | 0.05 | 0.02 | 0.19 | 0.08 | >100 |
| 21 | L-Ala OBn | L-Ala OBn | 6OMe2'CMeG | 0.49 | | 1.60 | | >100 |
| 22 | L-Ala OSPhEt | L-Ala OSPhEt | 6OMe2'CMeG | 0.49 | | 1.70 | | 88 |
| 23 | L-Ala O-2,4-diFBn | L-Ala O-2,4-diFBn | 6OMe2'CMeG | 0.26 | 0.21 | 1.07 | 0.92 | 54 |
| 24 | L-Ala OTHP | L-Ala OTHP | 6OMe2'CMeG | 13.33 | 13.02 | 29.30 | 15.14 | >100 |
| 25 | L-Ala OIndanol | L-Ala OIndanol | 6OMe2'CMeG | 0.58 | 0.39 | 1.30 | 0.10 | 70 |
| 26 | L-Ala OCH ₂ tBu | L-Ala OCH ₂ tBu | 6OMe2'CMeG | 0.06 | 0.04 | 0.20 | 0.12 | >100 |
| 27 | L-Ala OCH ₂ iPr | L-Ala OCH ₂ iPr | 6OMe2'CMeG | 0.07 | 0.01 | 0.26 | 0.01 | >100 |
| 28 | L-Ala OCH ₂ cPropyl | L-Ala OCH ₂ cPropyl | 6OMe2'CMeG | 0.21 | 0.00 | 0.66 | 0.001 | >100 |
| 29 | D-Ala OCH ₂ tBu | D-Ala OCH ₂ tBu | 6OMe2'CMeG | 0.11 | 0.03 | 0.50 | 0.19 | >100 |
| 30 | L-Asp OMe | L-Asp OMe | 6OMe2'CMeG | 10.18 | 3.17 | >40 | | >100 |
| 31 | L-Asp OBn | L-Asp OBn | 6OMe2'CMeG | 0.61 | 0.08 | 1.90 | 0.14 | >100 |
| 32 | L-Gly OBn | L-Gly OBn | 6OMe2'CMeG | 0.60 | 0.13 | 1.80 | 0.09 | >100 |
| 33 | L-Gly OCH ₂ tBu | L-Gly OCH ₂ tBu | 6OMe2'CMeG | 0.13 | 0.02 | 0.60 | 0.20 | >100 |
| 34 | L-Leu OcHx | L-Leu OcHx | 6OMe2'CMeG | 0.45 | 0.52 | 2.00 | 2.15 | 20 |
| 35 | L-Leu OBn | L-Leu OBn | 6OMe2'CMeG | 0.38 | 0.02 | 1.31 | 0.21 | 39 |
| 36 | L-Leu OCH ₂ tBu | L-Leu OCH ₂ tBu | 6OMe2'CMeG | 0.47 | 0.15 | 2.40 | 0.71 | 27 |
| 37 | L-Ile OMe | L-Ile OMe | 6OMe2'CMeG | 5.17 | 1.94 | 29.5 | 2.66 | >100 |
| 38 | L-Ile OcHx | L-Ile OcHx | 6OMe2'CMeG | 4.00 | 0.47 | 7.10 | 1.16 | 14 |
| 39 | L-Ile OBn | L-Ile OBn | 6OMe2'CMeG | 0.40 | 0.11 | 1.56 | 0.10 | 24 |
| 40 | L-Ile OCH ₂ tBu | L-Ile OCH ₂ tBu | 6OMe2'CMeG | 2.90 | 0.33 | 6.30 | 1.36 | 15 |
| 41 | L-Met OcHx | L-Met OcHx | 6OMe2'CMeG | 0.60 | 0.17 | 2.60 | 0.11 | 51 |
| 42 | L-Met OBn | L-Met OBn | 6OMe2'CMeG | 0.25 | 0.04 | 0.77 | 0.24 | >100 |
| 43 | L-Met OCH ₂ tBu | L-Met OCH ₂ tBu | 6OMe2'CMeG | 2.22 | | 5.00 | | >100 |
| 44 | L-Phe OcHx | L-Phe OcHx | 6OMe2'CMeG | 0.50 | 0.59 | 2.00 | 2.20 | 25 |
| 45 | L-Phe OBn | L-Phe OBn | 6OMe2'CMeG | 0.32 | 0.14 | 1.35 | 1.07 | 67 |
| 46 | L-Phe OCH ₂ tBu | L-Phe OCH ₂ tBu | 6OMe2'CMeG | 0.05 | 0.02 | 0.21 | 0.12 | 24 |
| 47 | L-Pro OBn | L-Pro OBn | 6OMe2'CMeG | 0.52 | 0.21 | 1.80 | 0.23 | >100 |
| 48 | L-Pro OCH ₂ tBu | L-Pro OCH ₂ tBu | 6OMe2'CMeG | 0.81 | 0.05 | 2.00 | 0.21 | 56 |
| 49 | L-Val OcHx | L-Val OcHx | 6OMe2'CMeG | 2.50 | 0.35 | 10.00 | 0.72 | 20 |
| 50 | L-Val OBn | L-Val OBn | 6OMe2'CMeG | 0.12 | 0.04 | 0.66 | 0.35 | 49 |
| 51 | L-Val OCH ₂ tBu | L-Val OCH ₂ tBu | 6OMe2'CMeG | 0.72 | | 2.50 | | 32 |
| 52 | L-Tyr (tBu) OMe | L-Tyr (tBu) OMe | 6OMe2'CMeG | 0.11 | 0.04 | 0.50 | 0.10 | 89 |
| 53 | L-PhG OcHx | L-PhG OcHx | 6OMe2'CMeG | 0.32 | 0.00 | 1.80 | 0.04 | 18 |
| 54 | L-PhG OCH ₂ tBu | L-PhG OCH ₂ tBu | 6OMe2'CMeG | 0.27 | 0.03 | 1.50 | 0.09 | 24 |
| 55 | L-Val-L-Ala OCH ₂ tBu | L-Val-L-Ala OCH ₂ tBu | 6OMe2'CMeG | 0.54 | 0.26 | 2.40 | 0.46 | >100 |
| 56 | β-Ala OBn | β-Ala OBn | 6OMe2'CMeG | 3.80 | 2.03 | >10 | | >100 |
| 57 | butylamine | butylamine | 6OMe2'CMeG | 37.80 | 3.80 | >40 | | >100 |
| 58 | morpholine | morpholine | 6OMe2'CMeG | >100 | | >100 | | >100 |
| 59 | L-Ala OcPentyl | L-Ala OcHx | 6OMe2'CMeG | 0.04 | 0.01 | 0.16 | 0.03 | >100 |
| 60 | L-Ala OCH ₂ tBu | L-Ala OBn | 6OMe2'CMeG | 0.15 | 0.04 | 0.40 | 0.07 | >100 |
| 61 | L-Ala OCH ₂ tBu | L-Ala OtBu | 6OMe2'CMeG | 0.15 | 0.01 | 0.57 | 0.08 | >100 |
| 62 | L-Ala OCH ₂ tBu | L-Ala OcHx | 6OMe2'CMeG | 0.04 | 0.01 | 0.15 | 0.02 | >100 |
| 63 | L-Ala OCH ₂ tBu | L-Pro OMe | 6OMe2'CMeG | 0.49 | | 2.20 | | >100 |
| 64 | L-Ala OCH ₂ tBu | L-Val OMe | 6OMe2'CMeG | 0.17 | 0.06 | 0.69 | 0.15 | >100 |

Table 1. continued

| compd | AA ester/amine | AA ester/amine | nucleoside | EC ₅₀ (μM) | | EC ₉₀ (μM) | | CC ₅₀ (μM) |
|-------|----------------------------|----------------------------|------------|-----------------------|------|-----------------------|------|-----------------------|
| | | | | av | SD | av | SD | |
| 65 | L-Ala OBn | butylamine | 6OMe2'CMcG | 0.44 | 0.28 | 1.68 | 0.94 | >100 |
| 66 | L-Ala OCH ₂ tBu | butylamine | 6OMe2'CMcG | 0.41 | 0.28 | 1.50 | 0.75 | >100 |
| 67 | L-Ala OCH ₂ tBu | pentylamine | 6OMe2'CMcG | 0.11 | 0.02 | 0.52 | 0.05 | >100 |
| 68 | L-Ala OCH ₂ tBu | cyclopropylamine | 6OMe2'CMcG | 0.87 | 0.11 | 4.70 | 0.45 | >100 |
| 69 | L-Ala OCH ₂ tBu | BnNH | 6OMe2'CMcG | 0.43 | 0.02 | 1.90 | 0.11 | >100 |
| 70 | L-Ala OCH ₂ tBu | PhNH | 6OMe2'CMcG | 0.27 | 0.01 | 0.82 | 0.00 | >100 |
| 71 | L-Ala OCH ₂ tBu | NaphNH | 6OMe2'CMcG | 0.13 | 0.05 | 0.56 | 0.18 | >100 |
| 72 | L-Ala OCH ₂ tBu | diethylamine | 6OMe2'CMcG | 3.70 | | >10 | | >100 |
| 73 | L-Ala OCH ₂ tBu | pyrrolidine | 6OMe2'CMcG | 0.87 | 0.02 | 4.00 | 0.16 | >100 |
| 74 | L-Ala OcHx | L-Ala OcHx | 6OEt2'CMcG | 0.05 | 0.00 | 0.15 | 0.02 | 69 |
| 75 | L-Ala OBn | L-Ala OBn | 6OEt2'CMcG | 0.29 | 0.04 | 1.00 | 0.29 | 62 |
| 76 | L-Ala OCH ₂ tBu | L-Ala OCH ₂ tBu | 6OEt2'CMcG | 0.04 | 0.01 | 0.11 | 0.04 | 72 |

of the neopentyl alanine compound as a naphthyl ProTide (5),¹² we were keen to explore the methylene bridged family starting with the neopentyl-(26) and 2-methylpropyl-(27) compounds and also the methylene cyclopropyl-(28).

On the basis of our recent report that the 6-ethoxy group can well substitute for the purine 6-methoxy as a core nucleoside for anti-HCV ProTides,²¹ we applied the diamidate method to the O6-ethyl-2'-C-methylguanosine nucleoside, preparing the symmetrical cyclohexyl-(74), benzyl-(75), and neopentyl-(76) L-alanine analogues.

In aryl phosphoramidate ProTides, we have reported on several occasions the sometimes strong preference, for L-amino acids over D-analogues,²⁵ and all of the first examples reported here are L-amino acid derived. However, we did synthesize the neopentyl-D-alanine analogue 29 of 8.

Although L-alanine is generally strongly preferred, other natural and unnatural amino acids are also effective as ProTides,²⁶ and so we did vary the amino acid motif itself. An attempt was made to consistently select from the same three ester groups, neopentyl, cyclohexyl, and benzyl, for each new amino acid to facilitate comparison. Thus, symmetrical diamidates were prepared from dimethyl-(30) and dibenzyl-(31) L-aspartic acid, benzyl-(32) and neopentyl-(33) glycine, cyclohexyl-(34), benzyl-(35), and neopentyl-(36) L-leucine, methyl-(37), cyclohexyl-(38), benzyl-(39), and neopentyl-(40) L-isoleucine, cyclohexyl-(41), benzyl-(42), and neopentyl-(43) L-methionine, cyclohexyl-(44), benzyl-(45), and neopentyl-(46) L-phenylalanine, benzyl-(47) and neopentyl-(48) L-proline, and cyclohexyl-(49), benzyl-(50), and neopentyl-(51) L-valine. The tyrosine methyl ester diamidate was also prepared as its *para*-O-*tert*-butyl ether-(52). Diamidates of the unnatural amino acid L-phenylglycine were prepared as its cyclohexyl-(53) and neopentyl-(54) esters. In a prior program on ProTides of d4T for HIV, we reported a complete loss of activity on extending from alanine to β-alanine and beyond,²⁷ but it was unclear whether similar restrictions would apply here, hence we prepared the symmetrical benzyl-β-alanine diamidate-(56).

Finally, in this series, we wondered if the chemistry methodology would extend to a dipeptide and whether such an adduct might be active and so we successfully incorporated an L-valyl-L-alanine neopentyl ester (55).

Simple amines have not been found to be useful as the amino component of aryl phosphoramidates, although Idenix has successfully incorporated benzylamine into their clinical analogue IDX184.¹⁴ However, for diamidates, the SAR was

unknown, and so we prepared the symmetrical butylamine-(57) and morpholinyl-(58) diamidates. Notably, from a synthetic perspective, none of the above amino acid and amine variations presented particular challenges although the yields from these reactions were not high and remain unoptimized.

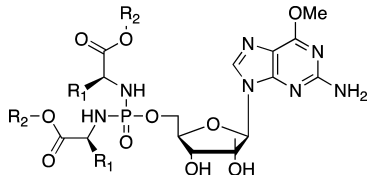
Besides seeking to probe the SAR and potential advantages of symmetrical diamidates, as above, we also wondered if the synthetic method would allow access to asymmetrically mixed diamidates. To do this, we slightly adapted the synthetic route (Scheme 1) to allow the stepwise introduction of two separate amino acids or one amino acid and one amine. To limit the large number of possible combinations, and to facilitate interpretation of the data, one of the amines was generally kept constant as neopentyl L-alanine and variations were made in the second amine. Initially, different L-alanine esters, benzyl-(60), *tert*-butyl-(61), and cyclohexyl-(62), were combined with neopentyl L-alanine, followed by different amino acids methyl L-proline-(63) and methyl L-valine-(64), and then a number of different simple amines (65–73). Synthetically, it did not matter much which amine was introduced first. Each of these asymmetric diamidates was isolated as a roughly 1:1 mixture of phosphate diastereoisomers as revealed by ³¹P NMR and HPLC. No attempt was made to separate the diastereoisomers, and they were tested as mixtures.

In this way, a substantial set of symmetrical and asymmetric phosphoramidates of 6-O-methyl-2'-C-methylguanosine and three derivatives of the 6-O-ethyl analogue were prepared and fully characterized. As expected, all of the symmetrical diamidates reported above were observed as one peak by ³¹P NMR and one signal on analytical HPLC. The ³¹P NMR shifts of the symmetrical amino aryl diamidates were ca. 13 ppm, being rather downfield of our usual aryloxy phosphoramidate ProTides.²⁸ The asymmetric diamidates gave two peaks by ³¹P NMR and for HPLC, in roughly 1:1 ratios, as typically observed for phosphoramidate ProTides. Other spectroscopic and analytical data fully confirmed the structure and purity of the diamidates herein reported.

■ BIOLOGICAL ACTIVITY IN REPLICON

Each of the diamidates described above were tested for HCV inhibition in a replicon assay, with the clinical anti-HCV ProTide (5) as positive control (Table 1). Both EC₅₀ and EC₉₀ values are reported, along with standard deviations, in a 72 h HCV replicon assay. In general EC₉₀ values were 2–5-fold higher than EC₅₀ values, and the discussion below will focus on

Table 2. Replicon Activity for Symmetrical Phosphorodiamidates



| amino acid | compd | replicon EC ₅₀ (μM) | | | CC ₅₀ in Huh7 cells (μM) |
|------------------|-------|--------------------------------|--------|-------------------|-------------------------------------|
| | | (R ₂) | | | |
| | | cyclohexyl | benzyl | neopentyl | |
| L-alanine | 20 | 0.05 | | | >100 |
| | 21 | | 0.49 | | >100 |
| | 19 | | | 0.06 | >100 |
| D-alanine | 29 | | | 0.11 | >100 |
| glycine | 32 | | 0.60 | | >100 |
| | 33 | | | 0.13 | >100 |
| L-leucine | 34 | 0.45 ^a | | | 20 |
| | 35 | | 0.38 | | 39 |
| | 36 | | | 0.47 | 27 |
| L-isoleucine | 38 | 4.0 | | | 14 |
| | 39 | | 0.40 | | 24 |
| | 40 | | | 2.9 | 15 |
| L-valine | 49 | 2.5 | | | 20 |
| | 50 | | 0.12 | | 49 |
| | 51 | | | 0.72 ^b | 32 |
| L-proline | 47 | | 0.52 | | >100 |
| | 48 | | | 0.81 | 56 |
| L-methionine | 41 | 0.60 | | | 51 |
| | 42 | | 0.25 | | >100 |
| | 43 | | | 2.22 ^b | >100 |
| L-phenylglycine | 53 | 0.32 | | | 18 |
| | 54 | | | 0.27 | 24 |
| L-phenylalanine | 44 | 0.50 ^a | | | 25 |
| | 45 | | 0.32 | | 67 |
| | 46 | | | 0.05 | 24 |
| L-aspartic (OBn) | 31 | | 0.61 | | >100 |

^aLarge standard deviation. ^bSingle assay result.

the EC₅₀ numbers. In addition, the cell cytotoxicities (CC₅₀) in the replicon cell line (Huh7) are reported.

As noted in Table 1, the three parent nucleosides, 2'-C-methyl guanosine **4** and the 6-methoxy **8** and 6-ethoxyl **9** analogues, display only modest anti-HCV activity, with EC₅₀ values in the 2–10 μM range. By comparison, **5** is active at nanomolar levels, with an EC₅₀ of 10 nM and an EC₉₀ of 40 nM, representing a ca. 400-fold potency boost over the respective nucleoside.¹² Compound **5** does show some cytotoxicity to Huh7 cells in this assay at 7 μM, but its high potency still leads to a significant SI of ca. 700.

Examining the potency of the first family of symmetrical L-alanine phosphorodiamidates, we note a consistent and clear

increase in potency for the *n*-alkyl esters as they extended from methyl (**10** EC₅₀ = 6 μM) to *n*-pentyl (**14** EC₅₀ = 0.03 μM). The *n*-pentyl L-alanine **14** is thus about 3 times less active than **5**, but notably it is also about 10 times less cytotoxic. It is interesting to note that the calculated lipophilicity (ClogP) values for this series range from 0.5 (methyl) to 4.7 (*n*-pentyl). It maybe that lipophilicity and potency correlate, however, there are exceptions. Clearly, the *n*-pentyl **14** is an interesting compound with a very attractive SI.

Branching the amino acid ester at the α (Table 1, **15**, **16**), β (**26**, **27**), or γ (**17**) position does not significantly change activity or toxicity relative to the straight chain compounds. Thus, the isopropyl ester **15** and the *n*-propyl ester **12** have

similar activities, and the β branched neopentyl ester **26** is similar to the *n*-pentyl ester **14**. Indeed, several of these branched esters such as compounds **17** and **27** are very potent, with excellent SI values. We were particularly interested in the neopentyl L-alanine analogue **26** given its similarity to our clinical agent **5**. This compound reveals an EC_{50} of $0.06\ \mu\text{M}$ and EC_{90} of $0.2\ \mu\text{M}$ and is only 5-fold less active in this assay than **5**. However, in common with many of the phosphorodiamidates, this compound is significantly less cytotoxic, with $CC_{50} > 100\ \mu\text{M}$. Thus, the SI for **26** is >1600 , which exceeds that of **5**.

Cyclic esters of L-alanine such as cyclopentyl-(**19**) and cyclohexyl-(**20**) are also very potent in the replicon assay and show no Huh7 cell toxicity at $100\ \mu\text{M}$. However, the cyclobutyl derivative **18** is about 5-fold less active with an EC_{50} of $0.32\ \mu\text{M}$, and the tetrahydropyranyl ester **24** is more than 200-fold less active compared to **20**.

Interestingly, the benzyl ester derivatives of L-alanine (Table 1, **21–23**) are somewhat less active than many of the alkyl ester analogues, being 5–10-times less active than the cyclohexyl compound for example. This is in marked contrast to the phosphoramidate ProTides SAR and much of our prior experience.²⁸

An observation from our phosphoramidate SAR is that the purine C-6-substituent can be varied considerably and that the 6-ethoxy may be particularly effective.²⁴ As noted in Table 1, the same applies to this series, with the cyclohexyl-(**74**), benzyl-(**75**), and neopentyl-(**76**) purine C-6-ethoxy derivatives all being equipotent with their C-6-methoxy analogues. Indeed, with an EC_{90} of $110\ \text{nM}$, the neopentyl L-alanine analogue of the 6-ethoxy nucleoside **76** emerged as one of the most potent compounds in the present study.

The SAR next turned to symmetrical phosphorodiamidates with amino acids other than L-alanine. The subtlest change was to make the D-alanine analogue as its neopentyl ester **29**. In contrast to our earlier work on D-amino acids in aryl phosphoramidate ProTides,²⁵ here we see only a slight (ca. 2-fold) loss of activity. This again points to quite a new and separate SAR for these diamidates as compared to aryloxy phosphoramidates.

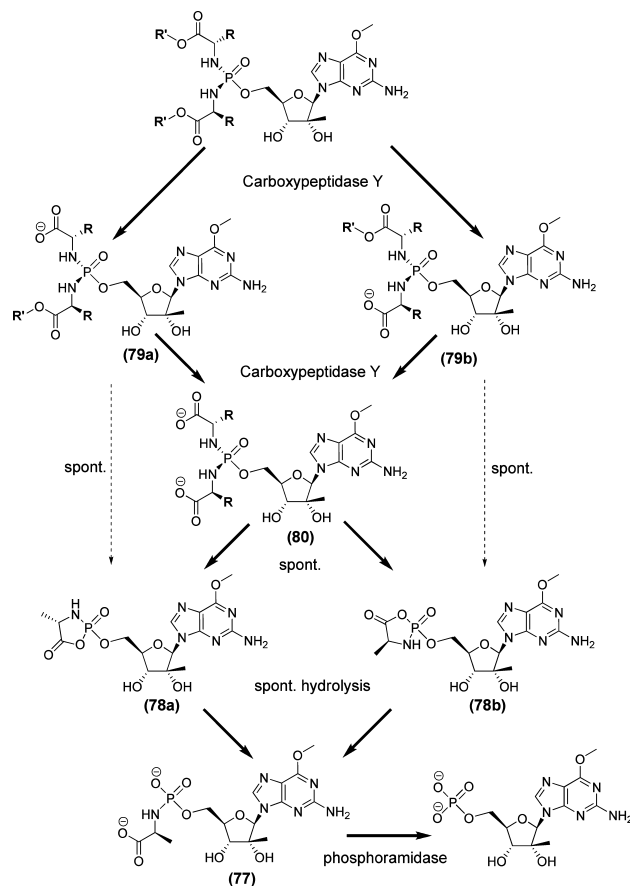
In generating the amino acid SAR in this series of symmetrically substituted phosphorodiamidates, effort was made to use three similar esters for each different amino acid derivative to facilitate comparison. The three ester groups selected were the cyclohexyl, benzyl, and neopentyl. Table 2 shows the compiled data.

A number of observations can be made from the data in Table 2. First, the benzyl esters do not greatly distinguish the different amino acids. The replicon activities for nine amino acids with benzyl ester groups range from 0.25 to $0.61\ \mu\text{M}$, only 2- to 3-fold, which is similar to the variability in the replicon assay.

The neopentyl and cyclohexyl ester groups distinguish the different amino acids and give a similar rank order. Thus, the best amino acid for both the cyclohexyl and the neopentyl esters is the L-alanine, whereas the worst is L-isoleucine.

Overall evaluation of the amino acid SAR leads to conclusion that small amino acid substituents like glycine or alanine are preferred and that increasing the size of the amino acid R_1 group as for L-leucine and L-methionine is detrimental to replicon activity, as is branching of the amino acid as for L-valine, L-isoleucine, and L-phenylglycine.

Scheme 2. Putative Initial Activation Route of Phosphorodiamidates



The exception to this rule is that the neopentyl L-phenylalanine derivative **46** is very potent ($EC_{50} = 0.05\ \mu\text{M}$) in the replicon assay. The cyclohexyl L-phenylalanine derivative **44** was tested twice, and one value was 10-fold higher than the other ($EC_{50} = 0.083$ and 0.92). It is possible that further testing would refine these data. Further supporting this surprising L-phenylalanine SAR, the L-tyrosine derivative **52**, as the *para*-*O*-*tert*-butyl ether, also had good activity and limited toxicity in the replicon assay ($EC_{50} = 0.11\ \mu\text{M}$, $CC_{50} = 89\ \mu\text{M}$).

A further observation in this series of symmetrical phosphorodiamidates is that some cytotoxicity is observed in Huh7 cells for the amino acids other than alanine and glycine. In fact, the cyclohexyl L-isoleucine **38** demonstrates that not all phosphorodiamidates are equivalent. Compound **38** has an EC_{50} of $4\ \mu\text{M}$ and a CC_{50} of $14\ \mu\text{M}$, for an SI of only ca. 4, and being the most cytotoxic diamidate studied.

As an extension of the growing symmetrical phosphorodiamidate SAR, a more complicated, dipeptide, L-Val-L-Ala neopentyl ester **55**, in which the L-valine nitrogen forms the phosphoramidate linkage, was prepared. Testing in the replicon assay shows that **55** has similar replicon activity (Table 1, $EC_{50} = 0.54\ \mu\text{M}$) as the neopentyl L-valine derivative **51**. It is unclear how this dipeptide would fit in with the putative metabolic process (see Scheme 2) and may suggest the existence of another metabolic pathway leading to the triphosphate formation for this compound.

Working the SAR in the opposite direction, simple amine substituted phosphorodiamidates were tested in the replicon assay. The *n*-butyl amine analogue **57**, which may be

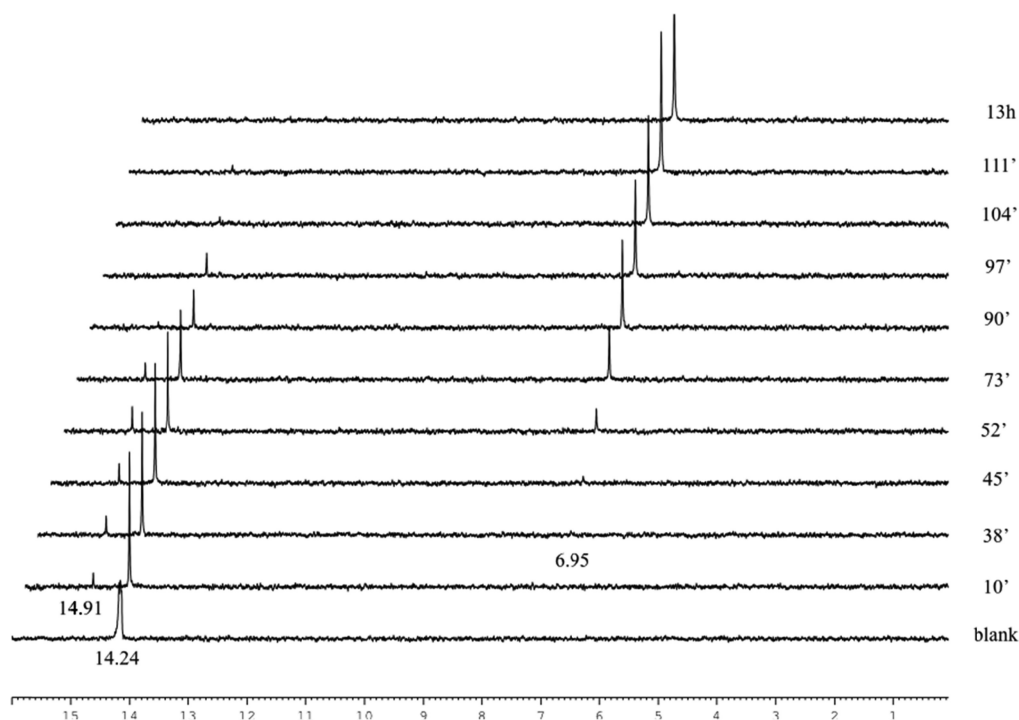


Figure 3. ^{31}P NMR kinetic study of **26** in the presence of carboxypeptidase Y. Conditions: 5.0 mg of **26** in 200 μL of acetone- d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3–0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

considered similar to the L-leucine derivatives, but without the α carboxylate, has a replicon EC_{50} of 38 μM , which is about 100-fold less active than the L-leucine derivatives. This is consistent with the proposed metabolic route (Scheme 2) where the carboxylate anion plays a key role. The positioning of the carboxylate β to the amine, as for the β -alanine analogue **56**, results in moderate activity in the replicon assay (Table 1, EC_{50} = 3.8 μM). The morpholino phosphorodiamidate **58**, an example of a secondary amine, is inactive even at 100 μM in the replicon assay. Both of these simple amine phosphorodiamidates **57** and **58** are much less active than the parent nucleoside **8** in the replicon assay, supporting our understanding that there is a limited direct cleavage of the P–O bond, leading to free nucleoside, in the replicon assay.

Although one of the key motivations for this new ProTide motif was to generate an achiral phosphate center, we were interested in understanding some aspects of the asymmetric diamidate SAR. Our synthetic route (Scheme 1) did allow the stepwise addition of two different amines. Each of these asymmetric diamidates were isolated as a roughly 1:1 mixture of phosphorus diastereoisomers, and no attempt was made to separate them and they were tested as mixtures. To facilitate comparison, generally, one of the two amines was maintained as the L-alanine neopentyl ester (Table 1).

From this basis, our SAR study was focused in three areas. First, we explored compounds **60**–**62** in which the second amine was also L-alanine but with different ester groups. The replicon results indicated that the presence of the L-alanine neopentyl ester provides good antiviral potency even in the presence of difficult to cleave²⁹ *tert*-butyl L-alanine ester **61**. The presence of two easily cleavable esters such as for derivatives **59** and **62** slightly increased the replicon activity comparing to **61** but one “cleavable” group was sufficient.

Next, the SAR of asymmetrical phosphorodiamidates, where the neopentyl L-alanine is combined with different amino acids

was briefly explored. The methyl L-proline **63** and the methyl L-valine **64** amino acids had moderate replicon activity (Table 1), more similar to the symmetrical L-prolines and L-valines than the very potent symmetrical neopentyl L-alanine **26**. It might be that compounds **63** and **64** have different metabolic intermediates (**78**, Scheme 2) than does **26**.

Third, the phosphorodiamidate SAR of simple amines combined with neopentyl L-alanine was extensively studied (**66**–**73**, Table 1). In general, these compounds had moderate to good activity, with EC_{50} values ranging from 0.1 to 0.9 μM for monosubstituted amines, more active than the symmetrical simple amines, but less active than the symmetrical neopentyl L-alanine derivative (**26**). Presumably, conversion of compounds **66**–**73** involves cleavage of the L-alanine neopentyl ester followed by the elimination of the simple amine to form the key metabolic intermediate (**78**, Scheme 2). Thus, it might be expected that the amines that make the best leaving groups such as phenylamine **70** and naphthylamine **71**, would be the most active (EC_{50} = 0.27 and 0.13 μM) and that a poor leaving group such as diethyl amine, in derivative **72**, would be the least active (EC_{50} = 3.7 μM , Table 1).

In conclusion, all but one of the 67 phosphorodiamidates tested in the replicon assay were active at or below micromolar concentrations, with 55 of them active below 1 μM and 12 active below 100 nM. Many are noncytotoxic at high μM levels, some at 100 μM , giving attractive SI values for many compounds. Clearly several of these compounds were worthy of advancing to in vivo studies.

■ STABILITY ASSAYS

Given the new structural motif we are reporting and the very promising replicon data (as described above), we sought to establish some outline stability data under a variety of conditions. To begin with, the lead symmetrical neopentylalanine compound **26** was dissolved in pH 7 phosphate buffer at

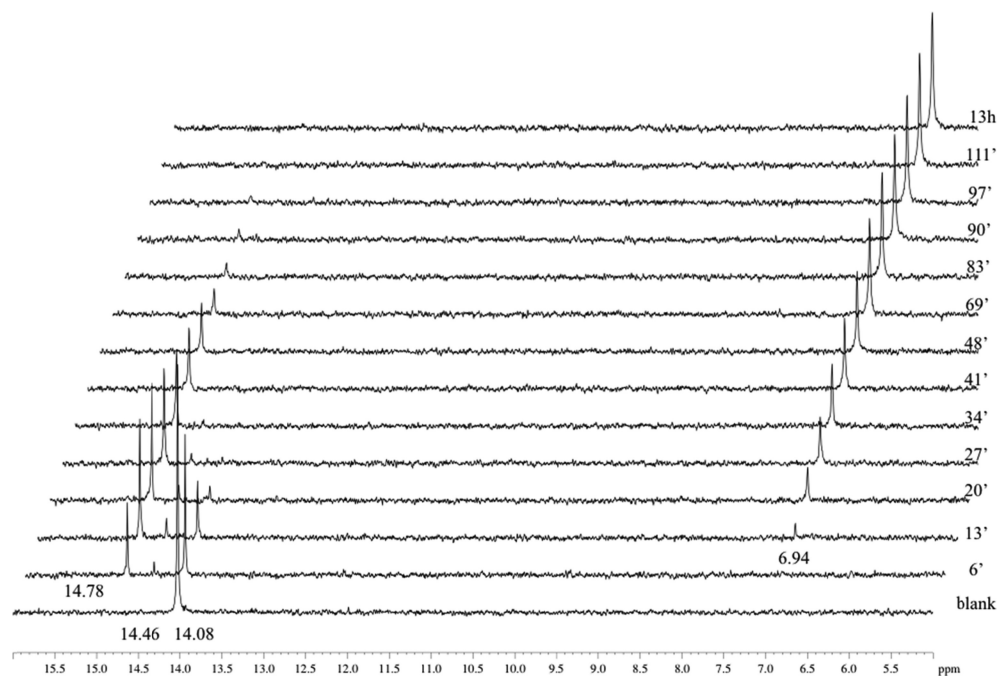


Figure 4. ^{31}P NMR kinetic study of **21** in the presence of carboxypeptidase Y. Conditions: 5.1 mg of **21** in 200 μL of acetone- d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3–0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

25 $^{\circ}\text{C}$ and monitored by ^{31}P NMR over 14 h. Compound **26** was observed at 13.2 ppm, and no additional signal(s) appeared over the course of the experiment (see Supporting Information for stability spectra). HPLC also indicated no detectable decomposition. We have previously reported acid stability data²⁷ for several acyclovir aryloxy phosphoramidate ProTides. As a representative example, the phosphorodiamidate (**26**) was dissolved in citric acid–HCl buffer at pH 2 and maintained at either 37 or 47 $^{\circ}\text{C}$ while being monitored by ^{31}P NMR over 14 h. Despite the acidic pH and elevated temperatures, the samples remained entirely stable over the course of the assay. A base stability study was conducted on compound **26** at pH 8.5/37 $^{\circ}\text{C}$ and pH 11/37 $^{\circ}\text{C}$, and these studies revealed slow decomposition. After 17 h at pH 11, the majority species was still unchanged (**26**), with only minor peaks at 6.9 ppm and –4.3 ppm. The peak at 6.9 ppm corresponds to an amino acid phosphoramidate derivative similar to compound **77** (see Scheme 2 below), suggesting one phosphorodiamidate P–N bond on compound **26** had been hydrolyzed under the aqueous basic conditions. However, the decomposition is rather slow, with a half-life exceeding 100 h (if first order). From this initial stability data, it seems possible that the phosphorodiamidates have a pH stability profile that is consistent with oral dosing.

Next, we studied the stability of compound **26** with human serum at 37 $^{\circ}\text{C}$. As before, the solution was monitored at 1 h intervals over by ^{31}P NMR over 12 h. The phosphorodiamidate **26** gave a peak at 14.5 ppm under these conditions, and no sign of degradation was observed by ^{31}P NMR. Thus, as noted for aryloxy phosphoramidates,³⁰ the present phosphorodiamidates seem essentially stable in human serum, certainly for periods of hours appropriate for human dosing.

As we have reported previously,^{22,30} the initial step of the conversion of aryloxy amino acid ester phosphoramidates (ProTides) to phosphates is thought to involve an enzyme-mediated cleavage of the amino acid ester group. We have also reported on the use of ^{31}P NMR and a buffered solution of the

enzyme carboxypeptidase Y as an in vitro model for studying the initial steps of ProTide³⁰ activation. We sought to apply this method to our new family of anti-HCV phosphorodiamidates. Thus, compounds of interest were dissolved in acetone- d_6 and TRIZMA buffer at pH 7.4, and the ^{31}P NMR spectrum was recorded as the baseline. Then carboxypeptidase Y (cathepsin) was added and spectra recorded at intervals up to 13 h (Figure 3). The data from these experiments were used to map a possible phosphorodiamidate metabolic pathway (Scheme 2).

The first compound studied was the symmetrical neopentyl ester of L-alanine **26**, which has a ^{31}P NMR shift of 14.2 ppm at baseline (Figure 3). After 10 min, a small downfield metabolite peak is observed at 14.9 ppm, which is consistent with cleavage of the neopentyl ester. The peak at 14.9 ppm builds up for approximately one hour, then diminishes, with a new peak emerging at 6.95 ppm. This is first observed at ca. 40 min and continues to grow through the course of the experiment. The peak at 6.95 corresponds to the key amino acid phosphate derivative **77** ($\text{R} = \text{CH}_3$) (Scheme 2). This was determined by synthesizing compound **77** ($\text{R} = \text{CH}_3$) and using it as an analytical reference standard (data not shown). Intermediate **77** could form by intramolecular attack of the amino acid carboxylate anion onto the phosphorus, with elimination of the second amino acid, followed by spontaneous hydrolysis of the cyclic, mixed anhydride intermediate **78a,b**. No direct ^{31}P NMR evidence (Figure 3) was obtained for this cyclic compound, consistent with it being a short-lived metabolic intermediate. As discussed in our publications on aryloxy amino acid phosphoramidates, final conversion to the nucleoside monophosphate is generally thought to be effected by enzymes of the histidine triad nucleotide binding (Hint) family (Scheme 2).^{30,33}

This initial evidence indicates that nucleoside phosphorodiamidates are converted to nucleoside monophosphates in a similar fashion as aryl phosphoramidates, that is, via metabolite **77**. To further support this hypothesis, a second phosphor-

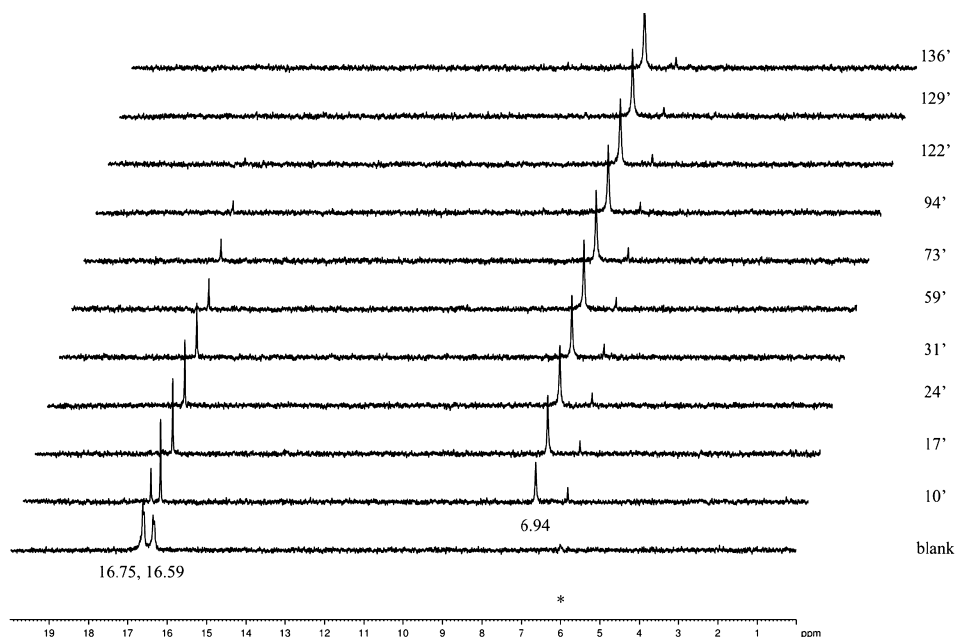


Figure 5. ^{31}P NMR kinetic study of **65** in the presence of carboxypeptidase Y. Conditions: 4.9 mg of **65** in 200 μL of acetone- d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3–0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4). * Phosphate impurity in Trizma buffer; in blank and remains unchanged throughout.

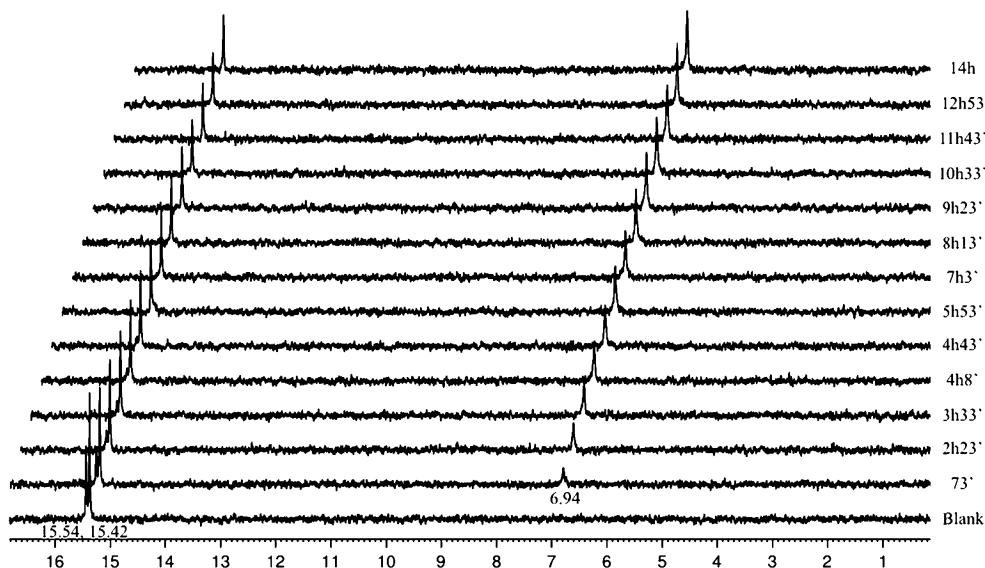


Figure 6. ^{31}P NMR kinetic study of **73** in the presence of carboxypeptidase Y. Conditions: 5.3 mg of **73** in 200 μL of acetone- d_6 + 400 μL of Trizma buffer (pH 7.4), 0.3–0.5 mg of carboxypeptidase Y in 200 μL of Trizma buffer (pH 7.4).

odiamidate, the symmetrical benzyl L-alanine derivative **21**, was studied in the same system (Figure 4). In this case, a similar ^{31}P NMR pattern, with the formation of the same key metabolite at 6.94 ppm (**77**, $\text{R} = \text{CH}_3$) is observed, but there are subtle differences. Parent **21** shows one phosphorus ^{31}P NMR peak at 14.08 ppm at baseline, which disappears within the first 30 min upon incubation with enzyme. However, in this case, two downfield singlet peaks are observed, one small and transient at 14.46 ppm and the other larger and longer lived at 14.78 ppm. Three possibilities exist to explain these two new downfield peaks: (a) one specific benzyl ester is cleaved by carboxypeptidase Y to give either **79a** or **79b**, but not both, followed by a second ester cleavage to give **80**; (b) both pro-pR and pro-pS benzyl esters are cleaved by carboxypeptidase Y and both

79a and **79b** are observed, but cleavage of both benzyl esters does not occur; (c) the peak at 14.46 ppm represents mono ester cleavage, where the ^{31}P NMR signals of **79a** and **79b** overlap, and the peak at 14.78 represents diester cleavage to give **80**.

Regardless of how the amino acid ester groups are cleaved by this particular enzyme, the important information from the ^{31}P NMR experiments is that compound **21** goes through the same common intermediate **77** ($\text{R} = \text{CH}_3$) as does compound **26**.

In Supporting Information, we present further detail of the kinetics of appearance of each species in the carboxypeptidase Y mediated cleavage of **21**.

It should be noted that **26** is nearly 10-fold more active than **21** in the HCV replicon assay (Table 1), however comparison

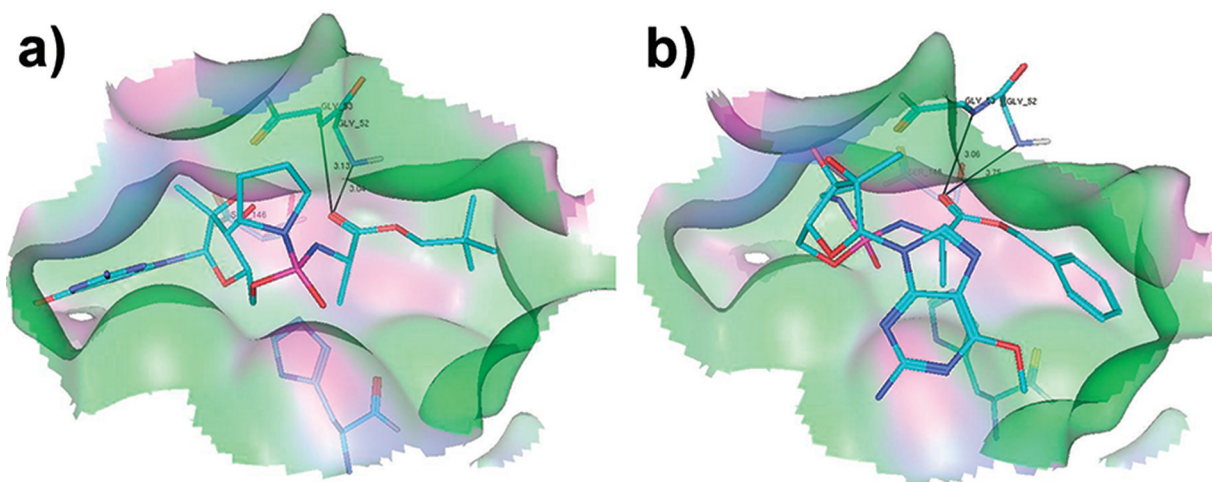


Figure 7. (a) Docking of compound **65-Rp** isomer within the catalytic site of carboxypeptidase Y. (b) Docking of compound **65-Sp** isomer within the catalytic site of carboxypeptidase Y.

Table 3. Rat Liver Triphosphate Levels from PK Analysis of Phosphorodiamidates

| compd | AA ester | replicon EC ₅₀ (μM) | C _{max} ^a (ng/g) | C _{last} ^b (ng/g) | T _{max} ^c (h) | AUC _{0-t} ^d (ng·h/g) |
|----------------|--------------------------------|--------------------------------|--------------------------------------|---------------------------------------|-----------------------------------|--|
| 5 ^e | L-Ala OCH ₂ tBu | 0.01 | 1446.7 | 983.3 | 8.0 | 24557 |
| 26 | L-Ala OCH ₂ tBu | 0.06 | 1580.0 | 800.0 | 8.0 | 25147 |
| 13 | L-Ala O- <i>n</i> -butyl | 0.07 | 1553.3 | 719.7 | 8.0 | 27671 |
| 14 | L-Ala O- <i>n</i> -pentyl | 0.03 | 1042.3 | 506.0 | 8.0 | 18600 |
| 16 | L-Ala O-(<i>R,S</i>)-2-butyl | 0.15 | 907.7 | 538.7 | 8.0 | 16252 |
| 19 | L-Ala O-cyclopentyl | 0.06 | 1523.3 | 610.0 | 8.0 | 24546 |
| 25 | L-Ala O-2-indanol | 0.58 | 632.0 | 632.0 | 24.0 | 10642 |
| 29 | D-Ala OCH ₂ tBu | 0.11 | 598.3 | 441.7 | 8.0 | 11608 |
| 50 | L-Val OBn | 0.12 | 359.5 | 73.6 | 4.0 | 4478 |

^aC_{max}: Maximum observed concentration. ^bC_{last}: Concentration at the last measurable time-point. ^cT_{max}: Time at which maximum concentration was observed. ^dAUC_{0-t}: area under the concentration–time curve from time 0 to the last measurable concentration. ^eCompound 5 is a the phosphoramidate INX-189 (see Figure 2).

of Figures 3 and 4 shows that the speed of carboxypeptidase Y processing is very similar for the two prodrugs, and thus the replicon and enzyme assays do not correlated directly, but we do regard enzyme mediated ester cleavage as a useful tool to study our prodrugs in vitro.

If a carboxylate anion intermediate such as **79a**, **79b**, or **80** can eliminate an amino acid moiety to give intermediate **77**, we wondered if simple primary or secondary amines could likewise be eliminated from asymmetric phosphorodiamidates such as **65** and **73**. Figure 5 shows the ³¹P NMR traces for an experiment with the benzyl L-alanine, *n*-butyl amine derivative **65**, and carboxypeptidase Y.

Because **65** is asymmetrical, two peaks are seen in the baseline ³¹P NMR (16.59 and 16.75 ppm). Both peaks disappear within 120 min, but the diastereomer at 16.75 is cleaved faster. Once again, a peak at 6.94, identified as compound (**77**, R = CH₃), is observed growing in magnitude during the course of the experiment. A very similar pattern is observed in Figure 6, when the neopentyl L-alanine, pyrrolidine asymmetrical phosphoramidate **73** was incubated with carboxypeptidase Y. Again compound **77** (R = CH₃) is observed at 6.94 ppm and grows in over 14 h. Interestingly, in this case, only one of the two diastereomers of compound **73** (³¹P NMR δ = 15.54 ppm) is cleaved by carboxypeptidase Y over the course of the experiment.

Summarizing this portion of our work, we have built on our previous understanding that aryloxy groups are eliminated from

asymmetrical phosphoramidates containing an amino acid carboxylate by showing that a second amino acid or a primary amine or a secondary amine can also be eliminated to give the key intermediate **77**. The data on **21**, **26**, **65**, and **73** supports the notion that only one ester cleavage is necessary and that the second amine loss is rapid following the first ester cleavage.

Docking Studies. To further support the above, we conducted some docking studies on several asymmetric diamidates, using published³¹ crystal structure of carboxypeptidase Y. Thus, as shown in Figure 7a,b the Sp diastereomer of **65** binds significantly better than the Rp diastereomer.

In the case of the Sp isomer, the stabilization by H-bonding of two glycine residues (Gly52, 53) is notable and the nucleophilic active site of Ser146 is also well positioned. These docking data would then suggest that one diastereoisomer of **65** might be processed better than the other. On the basis of the clear kinetic difference noted above (Figure 5), perhaps the more downfield species in ³¹P NMR of **65** is the Sp isomer. It is interesting to wonder if the differing kinetics of metabolism here may lead to a difference in biological potency as we have noted in some cases for aryl phosphoramidates.⁵ However, we were unable to separate the compound **65** diastereomers to test this hypothesis.

Pharmacokinetics. The HCV replicon, stability, and carboxypeptidase Y data suggests that our new phosphorodiamidate prodrug strategy may be a promising means of delivering 2'-C-methylguanosine triphosphate into cells. The

next hurdle was to determine if this prodrug strategy works in vivo in a rodent. Because of our growing experience with phosphoramidates such as **5** in rats, we decided to continue with the rat as our initial PK model for the phosphorodiamidates. Eight symmetrical derivatives were selected for rat PK studies (13, 14, 16, 19, 25, 26, 29, and 50, Table 3) based on activity in the replicon assay and structural considerations. We focused on symmetrical diamidates because they are represented by single diastereoisomers, which would simplify further development. All but two were L-alanine derivatives because we wanted to fully explore the ability of these relatively simple derivatives to provide sufficient liver triphosphate levels.

Compounds were formulated in 95% Capmul MCM/5% Tween 80, and doses of 10 mg/kg were administered by oral gavage to male Sprague–Dawley rats. Liver samples were collected up to 24 h postadministration and were snap-frozen in liquid nitrogen. Liver concentrations of 2'-C-methylguanosine triphosphate were determined by LC-MS/MS. Results for these eight phosphorodiamidates are compared to **5** in Table 3.

The main observation is that all compounds tested produced significant levels of triphosphate in rat livers from a 10 mg/kg dose, further validating phosphorodiamidates as effective phosphate prodrugs. In addition, several of the phosphorodiamidates provide similar liver triphosphate exposures as the clinical compound **5**, which has shown efficacy against HCV in phase Ib clinical trials.²³ The amount of 2'-C-methylguanosine triphosphate necessary to achieve an EC₉₀ in the replicon assay can be determined by measuring triphosphate levels in the replicon cells upon incubation with a 2'-C-methylguanosine based inhibitor such as **5**. This EC₉₀ triphosphate level in cells can be extrapolated to EC₉₀ triphosphate levels in the liver, as measured in ng of triphosphate/gram of liver tissue. The value we have calculated is 243 pmol of triphosphate per gram of liver (equal to 131 ng/g).³² Thus, for all phosphorodiamidates tested, except for **50**, the level of triphosphate 24 h post dose, is several fold above 131 ng/g level necessary to achieve 90% inhibition of HCV replication (Table 3).

An important part of our evaluation of any new prodrug approach for delivering 2'-C-methylguanosine triphosphate, including these phosphorodiamidates, is measurement of systemic nucleoside (2'-C-methylguanosine, **4**) levels after oral dosing of the prodrug. Our desire is to limit the systemic exposure of this nucleoside. The plasma 2'-C-methylguanosine levels for these eight phosphorodiamidates were measured, and all but compound **13** had lower nucleoside C_{max} values than **5** (<100 nM), and it is only slightly higher (data not shown).

The neopentyl L-alanine diamidate **26** has both the highest C_{max} and C_{last} of any diamidate tested. Figure 8 shows rat liver triphosphate levels after a 10 mg/kg dose for the clinical compound **5** and for **26**. Triphosphate levels were measured at eight time points over 24 h. It is clear that both prodrug strategies produce similarly high levels of triphosphate. The *n*-butyl L-alanine ester **13** has the highest overall triphosphate AUC (Table 3), and the cyclopentyl ester **19** also has excellent AUC and C_{last} values. These compounds along with the *n*-pentyl ester **14** were advanced to monkey PK studies. A combination of rat PK, monkey PK, and preliminary rodent toxicology studies will be used to help select a clinical candidate. These additional studies will be reported elsewhere upon completion.

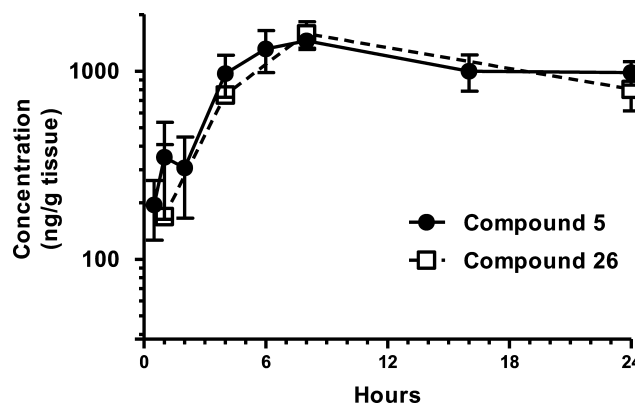


Figure 8. Rat liver 2'-C-methyl guanosine triphosphate levels from compounds **5** and **26**.

CONCLUSIONS

In conclusion, we report on a new family of phosphate prodrugs based on phosphorodiamidates. This type of 5'-monophosphate prodrug has the advantage that it can be designed to be achiral at the phosphate center if desired. We report on a range of novel prodrugs derived from amino acids and simple amines and build a substantial HCV replicon SAR for both symmetrical and asymmetrical phosphorodiamidates of 2'-C-methyl-6-O-methylguanosine. The replicon data suggests that one aminoacyl ester is essential for potent activity versus HCV. The phosphorodiamidates are stable in acid and mild base and also in human serum. Carboxypeptidase Y is able to activate these compounds to a nucleoside aminoacid phosphate key intermediate, which is also the essential metabolic intermediate for our earlier aryloxy ProTides. Many of the novel compounds in this study show low nanomolar activity versus HCV in replicon coupled with low cytotoxicity in the Huh7 replicon cell line. Eight potent HCV inhibitors were advanced to PK studies in Sprague–Dawley rats, and it was demonstrated that they all provided substantial 2'-C-methylguanosine triphosphate levels, in rat livers, that were maintained over a period of 24 h. This body of work has validated phosphorodiamidates as prodrugs for 2'-C-methylguanosine both at the in vitro and the in vivo levels. Further in vivo studies are underway that are intended to lead toward selection of a phosphorodiamidate prodrug for HCV clinical studies.

EXPERIMENTAL SECTION

General. Anhydrous solvents were purchased from Aldrich and used without further purification. All reactions were carried out under an argon atmosphere. Reactions were monitored with analytical TLC on silica gel 60-F254 precoated aluminum plates and visualized under UV (254 nm) and/or with ³¹P NMR spectra. Column chromatography was performed on silica gel (35–70 μM). Proton (¹H), carbon (¹³C), and phosphorus (³¹P) NMR spectra were recorded on a Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak, and all ¹³C NMR and ³¹P NMR were proton-decoupled. Analytical and semipreparative HPLC were conducted by Varian Prostar (LC Workstation-Varian prostar 335 LC detector) using Varian Polaris C18-A (10 μM) as an analytic column and Varian Polaris C18-A (10 μM) as a semipreparative column; elution was performed using a mobile phase consisting of water/acetonitrile in gradient (system 1, 90/10 to 0/100 v/v in 30 min) or water/methanol (system 2, 90/10 to 0/100 v/v in 30 min). High-resolution mass spectra (HRMS) was performed as a service by Cardiff University, using electrospray (ES). Compound purity was assured by a

combination of high field multinuclear NMR (^1H , ^{13}C , ^{31}P) and HPLC. Purity by the latter was always >95% with no detectable parent nucleoside for all final products.

Standard Procedure A: Synthesis Of Symmetrical Diamidates. To a suspension of the nucleoside (1.0 mol equiv) in anhydrous tetrahydrofuran, triethylamine (1.0–1.2 mol equiv) was added. After stirring for 30 min at room temperature, phosphoryl chloride (1.0–1.2 mol equiv) was added dropwise at -78°C . The reaction mixture was stirred for 30 min at -78°C and then allowed to warm to room temperature. Anhydrous dichloromethane was added, followed by amino acid ester (5.0 mol equiv) and triethylamine (10.0 mol equiv) at -78°C . After stirring at room temperature for 20 h, water was added and the layers are separated. The aqueous phase was extracted with dichloromethane and the organic phase washed with brine. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as an eluent a gradient of methanol in dichloromethane or chloroform. In some cases, a subsequent repurification was necessary either by preparative HPLC (gradient of methanol in water) or preparative TLC.

Standard Procedure B: Synthesis of Symmetrical Diamidates. To a solution of the nucleoside (1.0 mol equiv) in anhydrous triethylphosphate was added phosphoryl chloride (2.0 mol equiv) at 0°C . The reaction mixture was stirred for 24 h at 5°C . Anhydrous dichloromethane was added to the reaction mixture followed by amino acid ester (5.0 mol equiv) and diisopropylethylamine (10.0 mol equiv) at 0°C . After stirring at 5°C for 5 days, water was added and the layers were separated. The aqueous phase was extracted with dichloromethane and the organic phase washed with brine. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography using as an eluent a gradient of methanol in dichloromethane. A subsequent repurification, if necessary, was accomplished either by preparative HPLC (gradient of methanol in water) or preparative TLC.

Standard Procedure C: Synthesis of Asymmetrical Diamidates. To a suspension of the nucleoside (1.0 mol equiv) in anhydrous tetrahydrofuran, triethylamine (1.0 mol equiv) was added. After stirring for 30 min at room temperature, phosphoryl chloride (1.0 mol equiv) was added dropwise at -78°C . The reaction mixture was stirred for 30 min at -78°C and then allowed to warm to room temperature. Anhydrous dichloromethane was added, followed by the addition of amino acid ester or amine (1 mol equiv) and anhydrous triethylamine (2 or 1 mol equiv, respectively) at -78°C . Reaction was warmed to room temperature and monitored by ^{31}P NMR. When NMR indicated completion of the reaction (no starting material, presence of monosubstituted product), a second amino acid ester or amine (5 mol equiv) was added followed by the addition of triethylamine (10 or 5 mol equiv, respectively) at -78°C . After stirring at room temperature for 16–20 h, water was added and the layers were separated. The aqueous phase was extracted with dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue is purified by silica gel column chromatography using as an eluent a gradient of methanol in chloroform.

Example: Synthesis of 2-Amino-6-methoxy-9-(2'-C-methyl- β -D-ribofuranosyl) Purine 5'-O-Bis(methoxy-L-alaninyl) Phosphate (10). The phosphorodiamidate 10 was prepared according to the standard procedure B.

In a first step, a suspension of 6-O-methyl-2'-C-methylguanosine (250 mg, 0.803 mmol) in anhydrous tetrahydrofuran (4 mL) was reacted with triethylamine (135 μL , 0.964 mmol) and phosphorus oxychloride (89 μL , 0.964 mmol). In a second step, anhydrous dichloromethane (4 mL), L-alanine methyl ester hydrochloride salt (561.1 mg, 4.02 mmol), and triethylamine (1.12 mL, 8.03 mmol) were added.

After workup, silica gel column chromatography and preparative HPLC, 19.8 mg of (10) was obtained in 4.4% yield as an off-white solid. ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ 7.99 (s, 1H, H-8), 5.99 (s, 1H, H1'), 4.44–4.34 (m, 2H, H5'), 4.31 (d, 1H, J = 8.9 Hz, H3'),

4.21–4.16 (m, 1H, H4'), 4.07 (s, 3H, OCH_3), 3.98–3.89 (m, 2H, $2\times\text{CH}\alpha$ Ala), 3.70, 3.69 (2s, 6H, $2\times\text{OCH}_3$ ester), 1.33 and 1.32 (2d, 6H, J = 7.1 Hz, CH_3 Ala), 1.00 (s, 3H, CH_3).

^{13}C NMR (126 MHz, $\text{MeOD}-d_4$) δ 176.10 (2d, $^3J_{\text{C-C-N-P}}$ = 4.8 Hz, $2\times\text{C=O}$ Ala), 162.75 (C6), 161.95 (C2), 154.21 (C4), 139.33 (C8), 115.54 (C5), 93.19 (C1'), 82.33 (d, $^3J_{\text{C-C-O-P}}$ = 7.6 Hz, C4'), 80.02 (C2'), 74.73 (C3'), 66.13 (d, $^2J_{\text{C-O-P}}$ = 4.8 Hz, C-5'), 54.21 (OCH_3), 52.72 ($2\times\text{CH}_3$ ester), 51.01, 50.93 (2d, $^2J_{\text{C-N-P}}$ = 2.3 Hz, $2\times\text{CH}$ Ala), 20.86, 20.67 (2d, $^3J_{\text{C-C-N-P}}$ = 6.2 Hz, $2\times\text{CH}_3$ Ala), 20.27 ($2'\text{-CH}_3$). ^{31}P NMR (202 MHz, $\text{MeOD}-d_4$) δ 14.00. HPLC t_R = 8.86 min (system 1).

Example: Synthesis of 2-Amino-6-methoxy-9-(2'-C-methyl- β -D-ribofuranosyl) Purine 5'-O-Bis(benzoxy-L-alaninyl) Phosphate (21). The phosphorodiamidate 21 was prepared according to the standard procedure B.

In the first step, a solution of 6-O-methyl-2'-C-methylguanosine (250 mg, 0.803 mmol) in anhydrous triethylphosphate (1 mL) was reacted with phosphorus oxychloride (148 μL , 1.61 mmol). In the second step, anhydrous dichloromethane (4 mL), the tosylate salt of benzoxy-L-alanine (1.41 g, 4.02 mmol), and diisopropylethylamine (1.40 mL, 8.03 mmol) were added to the previous mixture. After workup, silica gel column chromatography and preparative HPLC, 50.1 mg of 21 was obtained in 8.7% yield as an off white solid. ^1H NMR (500 MHz, $\text{MeOD}-d_4$) δ 7.96 (s, 1H, H-8), 7.34–7.25 (m, 10H, $2\times\text{Ph}$), 5.99 (s, 1H, H1'), 5.16–5.02 (m, 4H, $2\times\text{CH}_2$ ester), 4.41–4.31 (m, 2H, H-5'), 4.29 (d, 1H, J = 9.0 Hz, H3'), 4.21–4.15 (m, 1H, H4'), 4.04 (s, 3H, OCH_3), 4.02–3.94 (m, 2H, $2\times\text{CH}$ Ala), 1.33 (d, 6H, J = 7.1 Hz, $2\times\text{CH}_3$ Ala), 0.99 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $\text{MeOD}-d_4$) δ 175.42, 175.36 (2d, $2\times\text{C=O}$, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, ester), 162.72 (C6), 161.91 (C2), 154.56 (C4), 139.31 (C8), 137.32, 137.29 (d, $2\times\text{C}$ ipso OCH_2Ph), 129.55, 129.5, 129.24, 129.21 (OCH_2Ph), 115.55 (C5), 93.17 (C1'), 82.37 (C4'), 80.01 (C2'), 74.81 (C3'), 67.89, 67.87 ($2\times\text{OCH}_2\text{Ph}$), 66.26 (C5'), 54.19 (OCH_3), 51.13, 51.08 (2d, $2\times\text{C}\alpha$ Ala), 20.79–20.58 (2d, $^3J_{\text{C-C-N-P}}$ = 6.3 Hz, $2\times\text{CH}_3$ Ala), 20.26 ($2'\text{CCH}_3$). ^{31}P NMR (202 MHz, $\text{MeOD}-d_4$) δ 13.93. HPLC t_R = 13.16 min (system 1).

Example: Synthesis of 2-Amino-6-methoxy-9-(2'-C-methyl- β -D-ribofuranosyl) Purine 5'-O-[(Benzoxy-L-alaninyl)-(2,2-dimethylpropoxy-L-alaninyl)] Phosphate (60). The phosphorodiamidate 60 was prepared according to the standard procedure C.

In the first step, a solution of 6-O-methyl-2'-C-methylguanosine (250 mg, 0.803 mmol) in anhydrous tetrahydrofuran (5 mL) was allowed to react with triethylamine (110 μL , 0.803 mmol) and phosphorus oxychloride (70 μL , 0.803 mmol). The tosylate salt of benzoxy-L-alanine (282 mg, 0.803 mmol) and triethylamine (110 μL , 0.803 mmol) were added. Anhydrous dichloromethane (4 mL) and the tosylate salt of neopentylglyoxy-L-alanine (1.33 g, 4.02 mmol) and triethylamine (1.12 mL, 8.03 mmol) were added as described in method C. After workup and silica gel column chromatography, 25 mg of the prodrug was obtained in 4% yield as an off-white solid. ^1H NMR (500 MHz, $\text{MeOH}-d_4$) 7.97, 7.96 (2s, 1H, H8), 7.36–7.30 (m, 5H, OCH_2Ph), 5.98, 5.97 (2s, 1H, H1'), 5.18–5.09 (m, 2H, OCH_2Ph), 4.39–4.33 (m, 2H, H5'), 4.28 (2d, J = 8.00 Hz, 1H, H3'), 4.20–4.16 (m, 1H, H4'), 4.06, 4.05 (2s, 3H, 6OCH_3), 4.02–3.94 (m, 2H, $2\times\text{CH}\alpha$ Ala), 3.84, 3.82, 3.72, 3.67 (2AB, J_{AB} = 10.50 Hz, 2H, $\text{CH}_2\text{C}(\text{CH}_3)_3$), 1.39–1.32 (m, 6H, $2\times\text{CH}_3$ Ala), 0.97 (s, 3H, $2'\text{CCH}_3$), 0.93, 0.91 (2s, 9H, $\text{CH}_2\text{C}(\text{CH}_3)_3$). ^{13}C NMR (126 MHz, $\text{MeOH}-d_4$) 175.54, 175.43, 175.39 (C=O ester), 162.73, 162.71 (C6), 161.93, 161.89 (C2), 154.57, 154.55 (C4), 139.32, 139.08 (C8), 137.39 (ipso OCH_2Ph), 129.55, 129.35, 129.25, 129.23, 129.20, 129.16, 128.27, 128.00 (OCH_2Ph), 116.19, 115.54 (C5), 93.34, 93.18 (C1'), 82.39, 82.33 (C4'), 80.01, 79.99 (C2'), 75.34, 75.04 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 74.84, 74.82 (C3'), 67.88, 67.85 (OCH_2Ph), 67.86 (d, $^2J_{\text{C-O-P}}$ = 3.75 Hz, C5'), 66.36 (d, $^2J_{\text{C-O-P}}$ = 5.50 Hz, C5'), 54.18, 54.01 (6OCH_3), 49.69, 49.64, 49.52, 49.46 ($2\times\text{C}\alpha$ Ala), 32.28, 32.25 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 26.74, 26.71 ($\text{CH}_2\text{C}(\text{CH}_3)_3$), 21.07, 20.90, 20.79, 20.66 (4d, $^3J_{\text{C-C-N-P}}$ = 6.25 Hz, $2\times\text{CH}_3$ Ala), 20.39, 20.25 ($2'\text{CCH}_3$). ^{31}P NMR (202 MHz, $\text{MeOH}-d_4$) 13.98, 13.94. HPLC t_R = 16.11, 16.80 min (system 1). MS (TOF ES+) m/z : 716.28 ($\text{M} + \text{Na}^+$, 100%). HRMS $\text{C}_{30}\text{H}_{44}\text{N}_7\text{O}_{10}\text{P}_1$ calculated, 694.2966; found, 694.2956

Biological Methods. Replicon Assays. The HCV inhibitory activity of compounds was evaluated in an Huh7 cell line expressing a stable, bicistronic subgenomic HCV genotype 1b (Con1) replicon encoding the Renilla luciferase reporter gene (Apath, LLC, Brooklyn, NY) as previously described.³² Cellular cytotoxicity was evaluated using the CellTiter-Glo Luciferase assay (Promega, Madison WI). A day before testing, 2×10^4 Huh7 cells were seeded in 96-well flat bottom white plates (Nunc, Roskilde, Denmark). Four-fold serial drug dilutions were made in growth medium and added to the cells. No drug controls were included in each plate. The plates were incubated in the presence of test compound for 3 days at 37 °C with 5% CO₂. Luciferase reagent was added to cells and plates were incubated for 20 min before measuring relative luminescent units (RLU) in a luminometer (Veritas, Turner Biosystems, Sunnyvale, CA).

Pharmacokinetic Studies in Rats. Rat studies were conducted at Inhibitex, Inc., in accordance with NIH Guidelines and following protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Inhibitex, Inc. Studies were carried out as previously described.³² Test compounds were formulated in 95% Capmul MCM (ABITEC Corp., Janesville, WI)/5% Tween 80 (Sigma, St. Louis, MO), and doses of 10 mg/kg were administered by oral gavage to male Sprague–Dawley rats (Taconic Farms, Germantown, NY). Liver samples were collected as a terminal procedure up to 24 h postadministration and were snap-frozen immediately upon collection in liquid nitrogen. Liver samples were stored frozen at ≤ -80 °C prior to analysis.

Bioanalysis of Pharmacokinetic Samples. The concentration of 2'-C-MeGTP in liver samples from rats was measured by LC-MS/MS as described previously.²⁸ The assay was linear ($r^2 \geq 0.99$) in the concentration range of 100–4000 ng per gram of tissue with $\geq 85\%$ accuracy and $\leq 2\%$ CV. Noncompartmental pharmacokinetic analyses were performed on the liver concentration data using WinNonlin v5.2 software (Pharsight, St. Louis, MO) as described previously.³²

■ ASSOCIATED CONTENT

■ Supporting Information

Preparative methods, spectroscopic and analytical data on target compounds plus ³¹P NMR stability assays and metabolic activation data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone/Fax: +44 29 20874537. E-mail: mcguigan@cardiff.ac.uk.

■ ACKNOWLEDGMENTS

We thank Helen Murphy for secretarial assistance.

■ ABBREVIATIONS USED

DAAs, direct acting antivirals; HCV, hepatitis C virus; AZT, 3'-azidothymidine; SAR, structure–activity relationships; TLC, thin layer chromatography; HPLC, high performance/liquid chromatography; ClogP, calculated logarithm of the octanol/water partition coefficient; PK, pharmacokinetics

■ REFERENCES

- (1) Soriano, V.; Madejon, A.; Vispo, E.; Labarga, P.; Garcia-Samaniego, J.; Martin-Carbonero, L.; Sheldon, J.; Bottecchia, M.; Tuma, P.; Barreiro, P. Emerging drugs for hepatitis C. *Expert Opin. Emerging Drugs* **2008**, *13*, 1–19.
- (2) Brown, N. A. Progress towards improving antiviral therapy for hepatitis C with hepatitis C virus polymerase inhibitors. Part 1: Nucleoside analogues. *Expert Opin. Invest. Drugs* **2009**, *18*, 709–725.
- (3) Balzarini, J.; Egberink, H.; Hartmann, K.; Cahard, D.; Vahlenkamp, T.; Thormar, H.; De Clercq, E.; McGuigan, C. Anti-

retrovirus specificity and intracellular metabolism of 2',3'-dideoxy-2',3'-dideoxythymidine (d4T, stavudine) and its d4T 5'-monophosphate triester prodrug. *Mol. Pharmacol.* **1996**, *50*, 1207–1213.

(4) Hecker, S. J.; Erion, M. D. Prodrugs of Phosphates and Phosphonates. *J. Med. Chem.* **2008**, *51*, 2328–2345.

(5) McGuigan, C.; Mehellou, Y.; Balzarini, J. Aryloxy Phosphoramidate Triesters: A Technology for Delivering Monophosphorylated Nucleosides and Sugars into Cells. *ChemMedChem* **2009**, *4*, 1779–1791.

(6) Drontle, D. P.; Wagner, C. R. Designing a pronucleotide stratagenic lessons from amino acid phosphoramidates of anticancer and antiviral pyrimidines. *Mini-Rev. Med. Chem.* **2004**, *4*, 409–419.

(7) McKenna, C. E.; Kashemirov, B. A.; Eriksson, U.; Amidon, G. L.; Kish, P. E.; Mitchell, S.; Kim, J.-S.; Hilfinger, J. M. Cidofovir peptide conjugates as prodrugs. *J. Organomet. Chem.* **2005**, 2673–2678.

(8) Hosteller, K. Y.; Stuhmiller, L. M.; Lenting, H. B. M.; Van den Bosch, H.; Richman, D. D. Synthesis and antiretroviral activity of phospholipid analogs of azidothymidine and other antiviral nucleosides. *J. Biol. Chem.* **1990**, *265*, 6112–6117.

(9) Perigaud, C.; Aubertin, A.-M.; Benzaria, S.; Pelicano, H.; Giradet, J.-L.; Maury, G.; Gosselin, G.; Kirn, A.; Imbach, I.-L. Equal inhibition of the replication of human immunodeficiency virus in human T-cell culture by ddA bis(SATE) phosphotriester and 3'-azido-2',3'-dideoxythymidine. *Biochem. Pharmacol.* **1994**, *48*, 11–14.

(10) Erion, M. D.; Reddy, K. R.; Boyer, S. H.; Matulich, M. C.; Gomez-Galeno, J.; Lemus, R. H.; Ugarkar, B. G.; Colby, T. J.; Schanzer, J.; Van Poelje, P. D. Design synthesis and characterization of a series of cytochrome P(450) 3A-activated prodrugs (HepDirect prodrugs) useful for targeting phosph(on)ate-based drugs to the liver. *J. Am. Chem. Soc.* **2004**, *126*, 5154–5163.

(11) Meier, C. 2-Nucleos-5'-O-yl-4H-1,2,3-benzodioxaphosphinin-2-oxides a new concept for lipophilic, potential prodrugs of biologically active nucleoside monophosphates. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 70–72.

(12) McGuigan, C.; Madela, K.; Aljarah, M.; Gilles, A.; Branciale, A.; Zonta, N.; Chamberlain, S.; Vernachio, J.; Hutchins, J.; Hall, A.; Ames, B.; Gorovits, E.; Ganguly, B.; Kolykhalov, A.; Wang, J.; Muhammad, J.; Patti, J. M.; Henson, G. Design, synthesis and evaluation of a novel double pro-drug: INX-08189. A new clinical candidate for hepatitis C virus. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4850–4854.

(13) Furman, P. A.; Wang, P. Y.; Niu, C. R.; Bao, D. H.; Symonds, W.; Nagarathnam, D.; Steuer, H. M.; Rachakonda, S.; Ross, B. S.; Otto, M. J.; Sofia, M. PSI-7851: A novel liver-targeting nucleotide prodrug for the treatment of Hepatitis C. *Hepatology* **2009**, *48* (Suppl), 59th Annual Meeting of the American Association for the Study of Liver Diseases, **2008**, San Francisco, CA, poster 1901.

(14) Zhou, X. J.; Pietropaolo, K.; Chen, J.; Khan, S.; Sullivan-Bolyai, J.; Mayers, D. Safety and pharmacokinetics of IDX184, a liver-targeted nucleotide polymerase inhibitor of hepatitis C virus, in healthy subjects. *Antimicrob. Agents Chemother.* **2011**, *1*, 76–81.

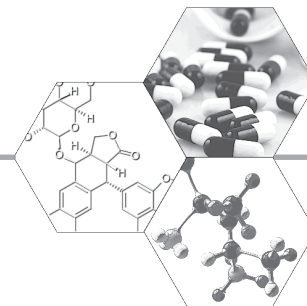
(15) Pengram, M.; Ku, N.; Shepard, M.; Speid, L.; Lenz, H.-J. Enzyme catalyzed therapeutic activation (ECTA) NB1011 (thymectacin) selectively targets thymidylate synthase (TS)-overexpressing tumor cells: Preclinical and phase 1 clinical results. *Eur. J. Cancer* **2002**, *38* (suppl, 7), 99.

(16) Congiatu, C.; Branciale, A.; Mason, M. D.; Jiang, W. G.; McGuigan, C. Novel Potential Anticancer Naphthyl Phosphoramidates of BVdU: Separation of Diastereoisomers and Assignment of the Absolute Configuration of the Phosphorus Center. *J. Med. Chem.* **2006**, *49*, 452–455.

(17) Jones, B. C. N. M.; McGuigan, C.; O'Connor, T. J.; Jeffries, D. J.; Kington, D. Synthesis and anti-HIV activity of some novel phosphorodiamidate derivatives of 3'-azido-3'-deoxythymidine (AZT). *Antivir. Chem. Chemother.* **1991**, *2*, 35–39.

(18) Serafinowska, H. T.; Ashton, R. J.; Bailey, S.; Harnden, M. R.; Jackson, S. M.; Sutton, D. Synthesis and in vivo evaluation of prodrugs of 9-[2-(phosphonomethoxy)ethoxy]adenine. *J. Med. Chem.* **1995**, *38*, 1372–1379.

- (19) Erion, M. D.; Van Poelje, P. D.; Dang, Q.; Kasibhatla, S. R.; Potter, S. C.; Reddy, M. R.; Reddy, K. R.; Jiang, T.; Lipscomb, W. N. MB06322 (CS-917): a potent and selective inhibitor of fructose 1,6-bisphosphatase for controlling gluconeogenesis in type 2 diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 7970–7975; *J. Hepatol.* **2011**, *54* (1), S187.
- (20) Nillroth, U.; Besidsky, Y.; Classon, B.; Chattopadhyaya, J.; Ugi, I.; Danielson, U. H. Specific interaction between HIV-1 proteinase and 5'-phosphate peptidomimetic derivatives of nucleoside analogs. *Drug Des. Discovery* **1995**, *13* (1), 43–54.
- (21) Nillroth, U.; Vrang, L.; Ahlsen, G.; Besidsky, Y.; Chattopadhyaya, J.; Ugi, I.; Danielson, U. H. The use of 5'-phosphate derivatives of nucleoside analogs as inhibitors of HIV-1 replication. *Antiviral Chem. Chemother.* **1995**, *6* (1), 50–64.
- (22) Romanowska, K. Biological activity and topological properties of HIV-1 inhibitors. *Pol. J. Chem.* **1993**, *67* (4), 755–757.
- (23) Barry, A.; Patti, J.; Matson, M.; Boehlecke, B.; Wenzel, E.; Pentikis, H.; Alam, J.; Henson, G. A Study of the Safety and Pharmacokinetics of Single Ascending Oral Doses of INX-08189 Nucleotide Polymerase Inhibitor, in Healthy Subjects. 46th European Association for the Study of the Liver, Berlin, Germany, March 30–April 3, 2011.
- (24) McGuigan, C.; Madela, K.; Aljarah, M.; Gilles, A.; Battina, S. K.; Ramamurty, C. V. S.; Srinivas Rao, C.; Vernachio, J.; Hutchins, J.; Hal, A.; Kolykhalov, A.; Henson, G.; Chamberlain, S. Dual prodrugs of 2'-C-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus. *Biomed. Chem. Lett.* **2011**, *21*, 6007–6012.
- (25) McGuigan, C.; Salgado, A.; Yarnold, C.; Harries, T. Y.; De Clercq, E.; Balzarini, J. Novel nucleoside phosphoramidates as inhibitors of HIV: studies on the stereochemical requirements of the phosphoramidate amino acid. *Antivir. Chem. Chemother.* **1996**, *7*, 184–188.
- (26) McGuigan, C.; Tsang, H.-W.; Cahard, D.; Turner, K.; Velazquez, S.; Salgado, A.; Bidois, L.; Naesens, L.; De Clercq, E.; Balzarini, J. Phosphoramidate derivatives of d4T as inhibitors of HIV: the effect of amino acid variation. *Antiviral Res.* **1997**, *35*, 195–204.
- (27) McGuigan, C.; Tsang, H.-W.; Sutton, P. W.; De Clercq, E.; Balzarini, J. Synthesis and anti-HIV activity of some novel chain extended phosphoramidate derivatives d4T (stavudine) esterase hydrolysis as a rapid predictive test for antiviral potency. *Antivir. Chem. Chemother.* **1998**, *9*, 109–115.
- (28) McGuigan, C.; Gilles, A.; Madela, K.; Aljarah, M.; Holl, S.; Jones, S.; Vernachio, J.; Hutchins, J.; Ames, B.; Bryant, D.; Gorovits, E.; Ganguly, B.; Hunley, D.; Hall, A.; Kolykhalov, A.; Liu, Y.; Muhammad, J.; Raja, Walters, N.; Wang, J.; Chamberlain, S.; Henson, G. Phosphoramidate ProTides of 2'-C-Methylguanosine as Highly Potent Inhibitors of Hepatitis C virus. Study of Their in Vitro and in Vivo Properties. *J. Med. Chem.* **2010**, *53*, 4949–4957.
- (29) McGuigan, C.; Sutton, P. W.; Cahard, D.; Turner, K.; O'Leary, G.; Wang, Y.; Gumbleton, M.; De Clercq, E.; Balzarini, J. Synthesis, anti-human immunodeficiency virus activity and esterase lability of some novel carboxylic ester-modified phosphoramidate derivatives of stavudine (d4T). *Antivir. Chem. Chemother* **1998**, *9*, 473–479.
- (30) Derudas, M.; Carta, D.; Brancale, A.; Vanpouille, C.; Lisco, A.; Margolis, L.; Balzarini, J.; McGuigan, C. The Application of Phosphoramidate Protide Technology to Acyclovir Confers Anti-HIV Inhibition. *J. Med. Chem.* **2009**, *52*, 5520–5530.
- (31) Endrizzi, J. A.; Breddam, K.; Remington, S. J. 2.8-A structure of yeast serine carboxypeptidase. *Biochemistry* **1994**, *33*, 11106–11120.
- (32) Vernachio, J. H.; Bleiman, B.; Bryant, K. D.; Chamberlain, S.; Hunley, D.; Hutchins, J.; Ames, B.; Gorovits, E.; Ganguly, B.; Hall, A.; Kolykhalov, A.; Liu, Y.; Muhammad, J.; Raja, N.; Walters, R. C.; Wang, J.; Williams, K.; Patti, J. M.; Henson, G.; Madela, K.; Aljarah, M.; Gilles, A.; McGuigan, C. INX-08189, a Phosphoramidate Prodrug of 6-O-Methyl-2'-C-Methyl Guanosine, is a Potent Inhibitor of Hepatitis C Virus Replication with Excellent Pharmacokinetic and Pharmacodynamic Properties. *Antimicrob. Agents Chemother.* **2011**, *55*, 1843–1851.
- (33) Chou, T.-F.; Baraniak, J.; Kaczmarek, R.; Zhou, X.; Cheng, J.; Ghosh, B.; Wagner, C. R. Phosphoramidate Pronucleotides: A Comparison of the Phosphoramidate Substrate Specificity of Human and *Escherichia coli* Histidine Triad Nucleotide Binding Proteins. *Mol. Pharmaceutics* **2007**, *4*, 208–217.



For reprint orders, please contact reprints@future-science.com

Progress in the development of anti-hepatitis C virus nucleoside and nucleotide prodrugs

The search for new anti-hepatitis C virus (HCV) therapeutics continues as the current treatment, consisting of PEGylated IFN- α and ribavirin, is of limited efficacy, nonspecific and can cause significant side effects. Modified nucleoside analogues with improved efficacy and selectivity, may become the backbone of the future standard of care for anti-HCV therapies. Several families of modified nucleoside are known to inhibit HCV RNA-dependent RNA polymerase, a vital enzyme for viral replication. Ongoing efforts are focused on improvement of potency, selectivity and delivery of antiviral nucleoside analogues, with several recent promising advances into clinical trials. This review summarizes the current progress in the development of new anti-HCV nucleoside and nucleotide prodrugs.

The **hepatitis C virus** (HCV) is one of the most important global healthcare burdens. Current estimates suggest that approximately 180 million individuals worldwide are chronically infected, with an anticipated 4 million new cases of infection each year [1–3]. A significant proportion of untreated patients are at risk of developing progressive liver diseases including cirrhosis, hepatocellular carcinoma and finally liver failure over a period of 25–30 years [4]. End-stage liver disease due to HCV infection represents the major indication for liver transplantation in the western world and related mortality has risen 2–5% per year [4,5].

There is no protective vaccination available against HCV. A lack of proper animal models has greatly limited the development of HCV vaccines. Currently, several different vaccines are in preclinical development with some advances into Phase I and II clinical trials. Their main mode of action is focused not only on antibody response but also on generation of cytotoxic CD8⁺ T cells [6].

HCV is a single stranded, positive-sense RNA virus belonging to the *Flaviviridae* family [7]. HCV replicates in the cytoplasm of liver hepatocytes and primarily targets cells [8]. Unrelenting infection relies on rapid production of viral particles, a high rate of mutation and a lack of effective T-cell immune response to HCV antigens [9]. Replication of the positive-stranded RNA genome is characterized by error rates of approximately 10^{-5} nucleotide substitutions per replicated base. With the estimated 12-log daily

virion production and turnover rate close to 99%, the calculated mutation rate is in the range of $1.5\text{--}2.0 \times 10^{-3}$ nucleotide substitutions per site, per year [10]. This results in a facile generation of genetic diversity, such that the virus exists as a quasi-species. Based on phylogenetic analysis, HCV has been classified into six major genotypes (represented by over 100 viral subtypes). Genotypes 1, 2 and 3 are predominant in the western world, Australia and east Asia, whilst types 4–6 are common in Africa, the Middle East and southeast Asia. HCV genotypes are associated with differing responses to antiviral therapy. Understanding of the clinical relevance and therapeutic implications of the HCV genotypes is crucial for designing individualized therapies for patients with chronic HCV infection [11,12].

Current treatment

The established standard of care (SOC) for patients infected with HCV is a combination therapy with PEGylated IFN- α (PEG-IFN) and the nucleoside analogue (NA) ribavirin (RBV) [13]. The antiviral activity of this dual regimen relies on enhancing the host's immune response [14]. The main goal of antiviral therapy for the hepatitis C infection is to achieve a sustained virological response (SVR) [15]. SVR is defined as achieving undetectable levels of HCV RNA (<50 IU/ml) in serum, at the end of a 24-week follow-up period post-therapy, and its continued absence for at least 6 months after stopping the treatment [16]. For patients infected with HCV genotype 1, SVR rates are highly

Karolina Madela & Christopher McGuigan*

Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff, CF10 3NB, UK

*Author for correspondence:
Tel.: +44 2920 874 537

E-mail: mcguigan@cardiff.ac.uk

**FUTURE
SCIENCE**

part of
fsg

Key Terms

Hepatitis C virus: Small, enveloped, positive-sense ssRNA virus, which is a member of the *Flaviviridae* family and the cause of hepatitis C infection in humans.

Nucleoside and nucleotide analogue inhibitors:

Nucleoside analogues that can bind to the active site of viral polymerase and compete with naturally occurring nucleoside triphosphates for incorporation.

Non-nucleoside inhibitors:

Antiviral agents acting at allosteric binding sites of the viral polymerase.

Prodrug: Pharmaceutical substance administered in an inactive or significantly less active form. A prodrug must undergo chemical conversion by metabolic processes before becoming an active pharmacological agent.

unsatisfactory. The response is achieved in approximately 34–52% treated patients after 48 weeks of therapy. Moreover, the therapy for HCV genotype 1-infected patients with relapse or nonresponsive to SOC is even more demanding, and the percentage of SVR is much lower. In comparison, for patients with HCV genotype 2 or 3 infections, SVR can be achieved in 81–84% after 24 weeks of dual therapy [15,17]. Furthermore, PEG-IFN/RBV therapy is often connected with side effects such as depression, anemia, flu-like symptoms, fatigue, cognitive dysfunction, cytopenias, thyroid dysfunction, retinopathy and others. Interferon is also administered as a subcutaneous injection, which is not preferred by patients [18].

Virological response rates have been shown to depend also on various host and viral factors such as age, weight, sex, race, liver enzymes, stage of fibrosis and HCV-RNA concentrations at the baseline [15].

To increase SVR rates, different treatment approaches are currently under investigation. For example, individualized therapy, including duration of treatment based on the HCV-RNA concentration at baseline, and trials with direct acting antiviral drugs such as inhibitors of HCV-specific NS3 protease and HCV NS5B RNA-dependent RNA polymerase (RdRp).

HCV genome

The HCV genome is approximately 9600 base pairs long and contains 5'- and 3'-noncoding regions. A single, open reading frame encodes a large polyprotein of 3010 amino acids. Host and viral proteases process this protein into three structural (Core, E1 and E2) and seven nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) individual, functional proteins (Figure 1) [19].

Most efforts in the development of novel HCV antiviral agents have focused mainly on two viral, nonstructural targets: the NS3 serine protease (responsible for cleavage of the HCV

proteins from the polyprecursor); and the NS5B RdRp, a crucial factor for HCV replication [20].

NS5B, the RdRp

Structural homology of RdRp across genotypes was found to be greater than 60%, with only 20–25% difference between subtypes [21]. The structure of NS5B consists of three subdomains referred to as palm, fingers and thumb [22]. Catalytic residues of the enzyme active site are located in the palm domain, including a Gly-Asp-Asp highly conserved motif [23]. Interactions between the fingers and thumb result in an almost completely encircled catalytic site. This encircling of the active site creates a channel that can accommodate the ssRNA but not dsRNA [23]. The two aspartic acid residues from the Gly-Asp-Asp motif, take part in the polymerization reaction allowing nucleotidyl transfer. They bind divalent metal ions (Mn^{2+} and Mg^{2+}) and coordinate the attack of the 3'-hydroxyl group of the growing RNA chain on the α -phosphate of the incoming nucleotide [22,23]. During the replication process, the negative strand of RNA serves as a template for the synthesis of the positive strand. The HCV polymerase is capable of starting the RNA synthesis *de novo*, by use of a single nucleotide primer, in particular guanosine 5'-triphosphate, which is the preferred initiating nucleotide [24,25].

NS5B inhibitors

HCV NS5B inhibitors can be classified into four classes based on their mode of action. The first class consists of **nucleoside and nucleotide analogue inhibitors** (NIs). NIs can bind to the active site of RdRp and compete with naturally occurring nucleoside triphosphates for incorporation. This class of inhibitors is likely to play a prominent role in the development of the new anti-HCV strategies. As the active site of NS5B is a highly conserved region for all six genotypes, NIs can exhibit pan-genotype inhibitory activity [26].

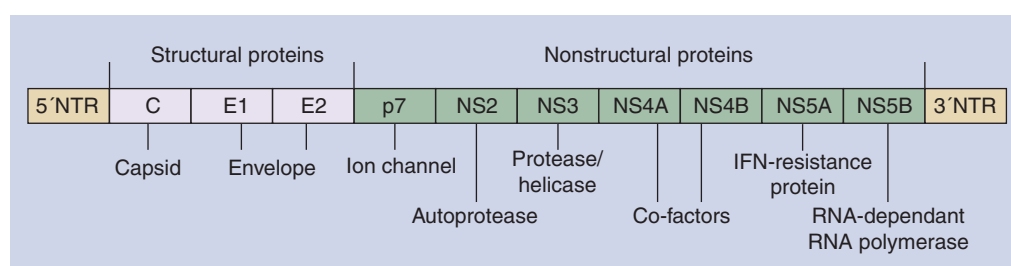


Figure 1. Organization of the hepatitis C virus genome.

The second group consists of **non-nucleoside inhibitors** (NNIs) acting at allosteric binding sites of the viral polymerase [27]. Pyrophosphate analogues represent a third class of inhibitors. They were designed to mimic the natural pyrophosphate released during the nucleotide transfer reaction and bind at the active site, disabling the binding of the next incoming nucleoside triphosphate [28]. Some of these inhibitors act by chelating the divalent metal ions required by the NS5B [29]. The fourth class consists of compounds targeting the cellular proteins required for HCV polymerase function [30].

NIIs are commonly designed to act as chain terminators. Obligate chain terminators lack a 3'-hydroxyl group on the sugar moiety and, therefore, the formation of the 3-5'-phosphodiester linkage is not possible. As a result, further extension of the viral RNA is stopped. A second class consists of non-obligate chain terminators. These nucleos(t)ides possess a 3'-hydroxyl group, however, other modifications of the sugar moiety cause conformational changes, sterically hindering the formation of subsequent phosphodiester bonds [31,201].

In general, triphosphates of 3'-deoxy-modified NAs were found to be potent chain terminators of the viral RdRp elongation *in vitro*. 3'-dCTP (**1**) and 3'-dGTP (**2**) were found to be amongst the most potent with K_i values of $0.72 \pm 0.20 \mu\text{M}$ and $0.93 \pm 0.08 \mu\text{M}$, respectively (**FIGURE 2**) [32].

Affinity of the modified nucleoside triphosphates (NTPs) to the RdRp was similar to that of the naturally occurring nucleoside triphosphates. Single nucleotide incorporation assays confirmed that 3'-deoxytriphosphate analogues could be incorporated with similar efficacy to substrates and could then act as chain terminators. However, when the corresponding nucleosides were tested in HCV subgenomic replicon assay, they were found to be poorly active, with EC_{50} values greater than $150 \mu\text{M}$. Further studies confirmed that this class of modified nucleosides suffers from poor activation into their triphosphate forms [32,33].

In the past decade, a number of ribonucleoside analogues with 2'-O-methyl, 2'-C-methyl and 4'-C-azido modifications have been reported to act as non-obligate chain terminators, specifically inhibiting HCV-RNA replication in replicon based assays. These classes of compounds, although potent and selective towards viral polymerase, suffer from poor bioavailability, liability to metabolic degradation and relatively poor turnover to the biologically active

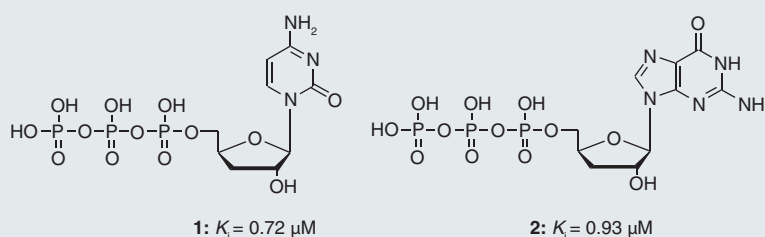


Figure 2. Structures of 3'-deoxy modified nucleoside analogue triphosphates with anti-hepatitis C virus RNA-dependent RNA polymerase activity.

K_i : Absolute inhibition constant.

triphosphates [34]. Several **prodrug** strategies, both at the nucleoside and nucleotide levels, have been implemented to overcome these limitations, and several specific reviews dealing with the topic have recently been published [26,35-38].

Nucleoside ester prodrugs

Several structurally distinct nucleoside inhibitors bearing either 4'-C-azido or 2'-C-methyl alterations have advanced into clinical trials, and simple ester prodrugs of these NAs have demonstrated improved pharmacokinetics and pharmacodynamics.

■ NM283

NM283 (valopicitabine; **3**) was the first HCV polymerase inhibitor to reach Phase IIb clinical trials. NM283 is an orally available prodrug of 2'-C-methylcytidine NM107 (**4**; **FIGURE 3**) [39].

2'-C-methylcytidine triphosphate is a potent inhibitor of NS5B with $\text{IC}_{50} = 0.09-0.2 \mu\text{M}$ (**5**; **FIGURE 3**) [40,41]; however, parent nucleoside **4** exhibited only modest activity ($\text{EC}_{50} = 2-7 \mu\text{M}$) when tested in replicon assay. Preliminary

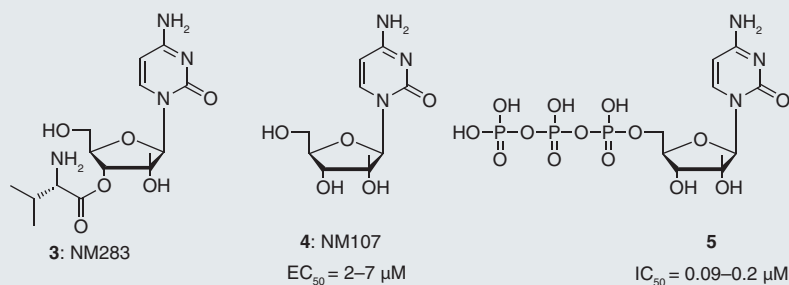


Figure 3. 2'-C-methylcytidine nucleoside NM107, triphosphate and 3'-valinyl ester prodrug NM283.

EC_{50} : 50% effective concentration or compound concentration required to inhibit hepatitis C virus replication by 50%; IC_{50} : 50% compound concentration required to inhibit activity of hepatitis C virus polymerase by 50%.

Key Term

ProTide: Pronucleotide, phosphorylated form of nucleoside analogues, which can be delivered intracellularly.

pharmacokinetic studies in animal models revealed low oral bioavailability of **4**. To overcome this limitation, the amino acid- 3'-*O*-L-valinyl ester of 2'-*C*-methylcytidine was prepared (**3**) and, as a result, oral bioavailability was enhanced [39]. NM283 was advanced into clinical trials. A Phase I study showed reduction in viral load by 0.7–1.0 log IU/ml after a 2-week treatment at a daily dose of 800 mg. Mild to moderate gastrointestinal adverse events were reported, with nausea and vomiting being the most common ones. A subsequent Phase IIb trial was carried out on treatment-naïve genotype 1 patients and nonresponders to a PEG-IFN/RBV regimen, over 48 weeks (200–800 mg/day). At week 24 an approximate 3.3 log IU/ml reduction of viraemia was demonstrated. Combination treatment with PEG-IFN (double regimen) led to a approximate 4.4 log IU/ml decline in HCV RNA after 36 weeks. The rapid viral response (RVR) was achieved in 18–49% of treated naïve patients by day 28, and 87–94% of infected individuals achieved early viral response by week 12. Initial HCV RNA decline for HCV genotype 1 nonresponders was poorer than in treatment-naïve patients (~2 log) using a PEG-IFN/NM283 regimen. Despite the fact that NM283 showed efficacy in clinical studies in both treatment-naïve patients and PEG-IFN/RBV nonresponders, when tested in combination with PEG-IFN the compound was discontinued from further development, due to the significant gastrointestinal toxicity [42–44].

■ R1626

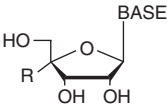
4'-*C*-azidocytidine R1479 (**6**) was discovered within the group of 4'-modified pyrimidine

analogues as a specific inhibitor of HCV replication without noticeable cytotoxicity and cytostatic properties. Most of the 4'-substituted analogues were poorly active and nontoxic in cell-based HCV replicon assays, apart from **6** and **13** (TABLE 1) [45].

The 5'-triphosphate of 4'-*C*-azidocytidine (**15**) was shown to inhibit viral NS5B polymerase with $IC_{50} = 0.29 \mu M$. 2',3',5'-tri-*O*-isobutyrate ester prodrug of 4'-*C*-azidocytidine (R1626) was selected for further development (**16**; FIGURE 4) [45].

The introduction of isobutyrate groups reduced the polarity of R1479 and enhanced uptake of **6** from the GI tract [45]. Due to the promising outcomes of the preclinical development, this compound was progressed into clinical trials. During Phase Ib, a dose-dependent reduction of viral load up to approximately 3.7 log IU/ml was observed in genotype 1 patients administered at a dose of 500–4500 mg/day. After a 2-week treatment, five out of nine patients treated with the highest dose had undetectable levels of viral RNA. Combination therapy with IFN- α and/or RBV gave an average of 4.2 log IU/ml reductions of HCV RNA, with 33–80% of patients achieving nondetectable levels of HCV RNA at week 4, compared with the 2.4 log IU/ml reduction in the placebo/PEG-IFN/RBV control group, and 5% of RVR within the control arm. Efficacy results of the Phase IIa clinical trials showed high and rapid clearance of the virus; however, high rates of Grade IV neutropenia (39–78%) and anemia (32%) were reported as dose-dependent side effects. Further development of R1626 was discontinued due to unspecified adverse events [46–48,301].

Table 1. Evaluation of 4'-substituted nucleosides.

|  | | | | |
|--|-----------------------------|------|---------------------------------------|--------------------------------|
| Compound | R | Base | Replicon inhibition at 20 μM (%) | Cytotoxicity at 20 μM (%) |
| 6 | N ₃ | C | 97 ($EC_{50} = 1.3 \mu M$) | 17 ($CC_{50} > 100 \mu M$) |
| 7 | N ₃ | U | 13 | 17 |
| 8 | Et | C | 20 | 20 |
| 9 | C \equiv N | C | 99 | 100 |
| 10 | C \equiv N | U | 0 | 0 |
| 11 | C \equiv CH | C | 3 | 0 |
| 12 | C \equiv CH | U | 0 | 0 |
| 13 | C \equiv CCH ₃ | U | 76 | 19 |
| 14 | C \equiv CCH ₃ | C | 11 | 0 |

CC_{50} : 50% cytotoxic concentration; EC_{50} : 50% effective concentration or compound concentration required to inhibit hepatitis C virus replication by 50%.

■ RG7128

RG7128 (mericitabine; **17**) is a 3',5'-*bis*-isobutyryl ester prodrug of PSI-6130 (**18**; **FIGURE 5**) [48].

β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130) was found to be a potent inhibitor of HCV replication in Huh7 replicon assay and its triphosphate (**19**; **FIGURE 5**) was found to be a selective inhibitor of NS5B *in vitro*, without any affinity towards human DNA and/or RNA polymerases [48]. PSI-6130 showed broad activity across all six HCV genotypes without apparent cytotoxicity [49]. The compound was advanced into clinical trials but showed only modest oral bioavailability and an unfavorable metabolic pathway leading to the uridine metabolite RO2433 [50]. To overcome these outcomes the *bis*-isobutyryl ester prodrug of PSI-6130 was synthesized (**7**; **FIGURE 5**). According to the published data, RG7128 is the most advanced, simple nucleoside prodrug in clinical trials [51]. During Phase I study, **17** has shown efficacy when tested in patients infected with HCV genotypes 1, 2 and 3. A 2.7 log IU/ml of viral load reduction was observed over 2-week monotherapy course (1500 mg b.i.d. dose). A 4-week combination Phase IIa trial of 1000 and 1500 mg b.i.d. doses of RG7128 with PEG-IFN/RBV in 81 genotype 1 treatment-naïve patients resulted in approximately 5.0 log IU/ml decline in viral RNA levels. The SOC control arm gave a 2.7 log IU/ml HCV RNA reduction resulting in an RVR of 19% versus 85% (88% when coadministered with RG7128). The RVR of genotype 2 and 3 relapse and/or nonresponders (25 patients, 1500 mg b.i.d. over 28 days) was found to be enhanced by up to 90% (5 log IU/ml reduction) versus 60% (3.7 log IU/ml decline) of SOC control arm [51,302].

Reported data indicate that the adverse events profile during a 28-day study was similar to the control arm (for all tested genotypes), with no serious adverse events and most adverse events being mild. Grade 4 neutropenia was reported in 5% of patients in the RG7128 cohort compared with 10% in the placebo group [302]. RG7128 was advanced into Phase IIb clinical trials. Early reported data (a 12-week analysis) indicate an average 83% of complete early virological response for the 1000 mg b.i.d./SOC cohort. RG7128 is expected to complete this study in the near future [302].

ProTides (nucleotide prodrugs)

As already mentioned, NAs can suffer in terms of variable aqueous solubility and bioavailability

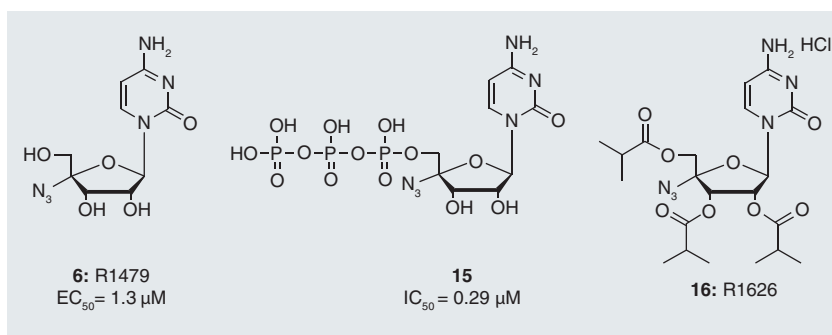


Figure 4. 4'-C-azidocytidine nucleoside R1479, 4'-C-azidocytidine triphosphate and nucleoside ester prodrug R1626. EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC₅₀: 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

following oral administration. Furthermore, NA activity depends upon metabolism to form sequentially the respective 5'-mono-, di- and triphosphates. Usually the first phosphorylation of NAs is a rate-limiting step (**FIGURE 6**) [52].

Therapies involving long-term administration of NAs may lead to decreased activity of the kinases responsible for the first phosphorylation step [53]. Administration of the preformed 5'-monophosphates would overcome the problem; however, at physiological pH the phosphates are negatively charged and, therefore, too hydrophilic to efficiently cross cell membranes. In addition, 5'-nucleotidases present in the blood and cell surface rapidly convert free nucleoside 5'-monophosphates to the corresponding nucleosides. Various pronucleotide approaches have been investigated in order to deliver masked 5'-monophosphates and these have improved the potential of NAs as therapeutics [54]. Successful **ProTides** would have to promote

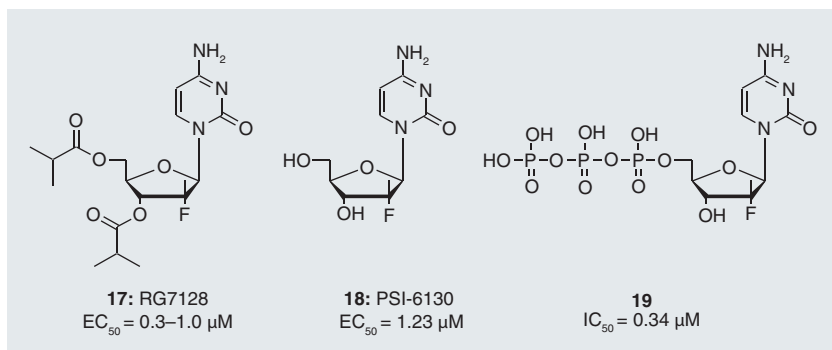


Figure 5. β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine – PSI6130, PSI6130 triphosphate and *bis*-isobutyryl ester prodrug, RG7128.

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC₅₀: 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

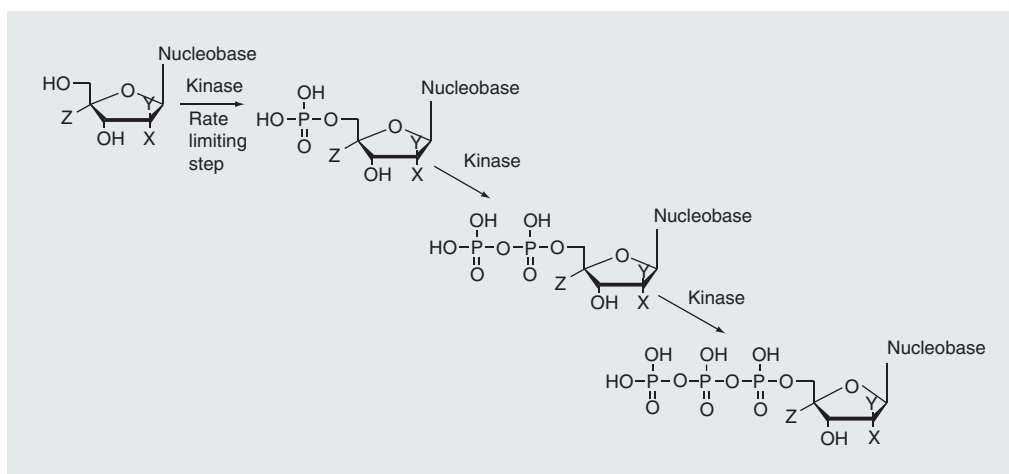


Figure 6. Phosphorylation pathway of nucleoside analogues.

passive diffusion through the cell membrane. They would need to possess reasonable chemical stability required for the formulation process. ProTides should be stable in gastrointestinal fluids and undergo an efficient first-pass metabolic pathway (for HCV activity). Once in hepatocytes, 5'-monophosphates would have to be successfully liberated and phosphorylated to the active triphosphate species. Several prodrugs of modified nucleotides have demonstrated proof of concept, delivering monophosphates into cells both *in vitro* and *in vivo* [56–58].

■ Aryloxy phosphoramidates

The first example of bioactive aryloxy phosphoramidates was reported by McGuigan *et al.* in 1992, as prodrugs of AZT (azidothymidine) [59]. Further development of the approach resulted in ProTides containing alkyl or haloalkyl esters in place of the aryl moiety and SAR studies of the amino acid side chain [60–62]. The phosphoramidate approach was later successfully

applied to a number of different NAs, greatly improving their antiviral or anticancer activity. ProTide technology was used for the following NAs: ddA, d4A [63], AZU [64], AZA [65], d4T [66,67], PMEA, PMPA [68], abacavir [69] and carbocyclic adenosine derivatives [70]. The application of phosphoramidate technology to *N*-acetyl glucosamine is the most recent and the first example of ProTides used on sugars [71]. The aryloxy phosphoramidate approach was found to be very effective in delivering masked monophosphates into cells. The release of monophosphate was found to be initiated by enzyme-mediated hydrolysis of the amino acid ester moiety [72,73], which generates a nucleophilic carboxylic acid moiety. Intramolecular nucleophilic attack on the phosphorus atom would give a putative unstable cyclic intermediate with release of the aryloxy moiety. The intermediate is then rapidly hydrolyzed followed by enzyme mediated P–N bond cleavage to yield nucleoside monophosphate (**FIGURE 7**) [66,67,74,75].

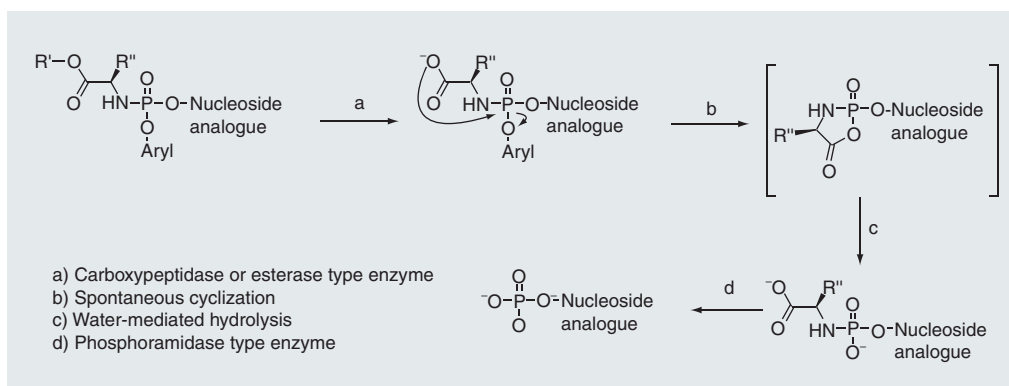


Figure 7. Possible mechanism of activation of aryloxy-phosphoramidates.

Wagner *et al.* developed phosphoramidate monoesters as a variation of the aryloxy phosphoramidate approach [76]. Phosphoramidate monoesters contain an amino acid ester as a masking group on one of phosphate charges. Application of only one masking group eliminates the chirality issue at phosphorus and provides better water solubility while simultaneously maintaining stability in human plasma. Although cellular permeation may be more limited, this kind of prodrug can represent an alternative method of nucleoside monophosphate delivery for intravenous (iv.) applications. Activation of phosphoramidate monoesters requires phosphoramidase activity to cleave the P–N bond and proceeds directly to the monophosphate [76].

■ S-acyl-2-thioethyl ProTides

The S-acyl-2-thioethyl (SATE) approach was firstly reported by Imbach and Gosselin. This strategy was successfully applied to different nucleosides such as ddU, ddA, dIdI, AZT, isoddA, d4T and ACV [77–80]. The bioenzymatic activation of bis(SATE) is initiated by the carboxyesterase cleavage of the thioester moiety of the SATE groups. The generated mercaptoethyl-phosphotriester is unstable and undergoes intramolecular nucleophilic attack, releasing ethylene sulphide and the monoSATE phosphodiester. The resultant phosphodiester is further hydrolyzed either by a phosphodiesterase or by carboxyesterase to release nucleoside 5'-monophosphate – this step is the same for mono- and cyclic-SATE prodrugs (**FIGURE 8**) [78]. The biggest concern of this class of compounds is the release of episulfide during activation. Episulfide is a highly potent alkylating agent and known mutagen; therefore, the toxicity risk associated

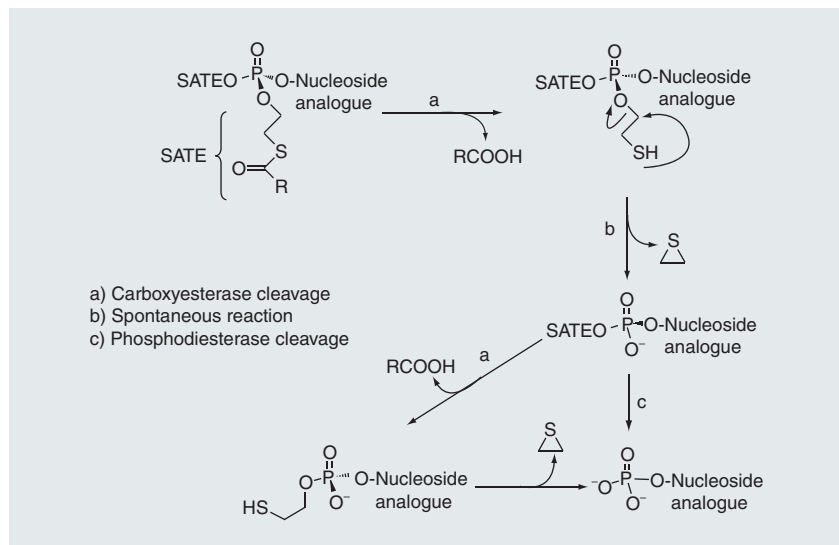


Figure 8. Mechanism of activation of bis(SATE) prodrug.

with it has mainly limited further clinical development [77].

Application of the bis(SATE) technology to the anti-HCV nucleosides was investigated. One of the reported examples of this approach is bis(SATE) prodrug of 2'- α -F-2'-C-methyl-7-ethynyl-7-deazadenosine [81]. The corresponding nucleoside triphosphate (**20**) was found to be an efficient chain terminator of the HCV NS5B polymerase with IC_{50} of 0.4 μ M. However, the parent nucleoside (**21**) had very low activity (24 μ M) in Huh7 replicon assay. Although delivery of masked 5'-monophosphate using the bis(SATE) approach (**22**) was successful and resulted in a modest increase of potency EC_{50} = 8 μ M, no additional studies of this family of pronucleotides have been reported (**FIGURE 9**) [81].

Benzaria *et al.* reported application of bis(t-BuSATE) technology to a 2'-C-methylcytidine

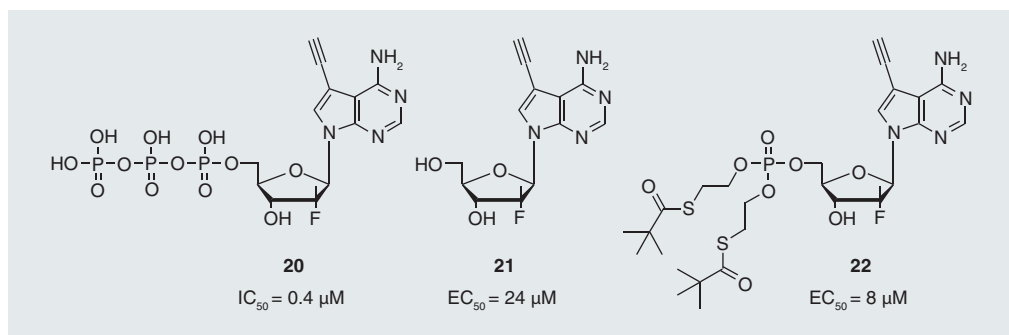


Figure 9. Structures and anti-hepatitis C virus activity of 2'- α -F-2'-C-methyl-7-ethynyl-7-deazaadenosine, corresponding triphosphate and S-acyl-2-thioethyl prodrug.

EC_{50} : 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC_{50} : 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

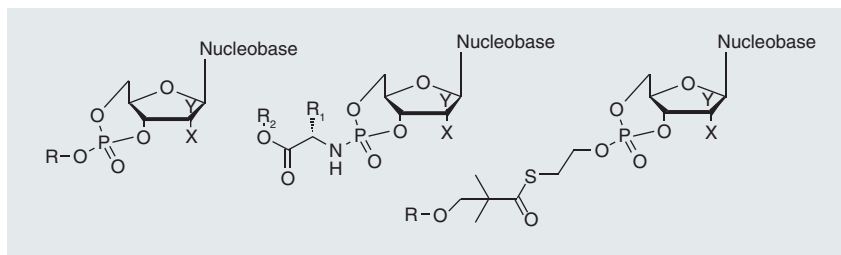


Figure 10. General structures of different 3',5'-cyclic ProTides.

and 2'-C-methyluridine. The corresponding SATE ProTides exhibited greatly improved potency against HCV compared with the parent nucleosides ($EC_{50} = 0.7 \mu\text{M}$ vs $EC_{50} = 2.2 \mu\text{M}$ for 2'-C-methylcytidine, and $EC_{50} = 0.1 \mu\text{M}$ vs $EC_{50} = 46 \mu\text{M}$ for 2'-C-methyluridine), however an increase in cytotoxicity was also observed [82].

Further evaluation of S-acyl-2-thioethyl ProTides resulted in the synthesis of cyclic- [83] and hybrid-SATE ProTides [84] with potent anti-HCV activity, to be discussed below.

■ Cyclic ProTides

The design of 3',5'-cyclic ProTides was based on naturally occurring cyclic monophosphates such as cyclic AMP [85]. Cyclisation of the phosphate moiety with the 3'-hydroxyl group was designed to decrease the ionic nature of phosphate and improve lipophilicity. It was postulated that the 5'-monophosphate would be released upon exposure to phosphodiesterases [86]. Several studies have shown that the cell uptake of different cyclic AMP analogues was not efficient, presumably due to the remaining charge on the phosphate center and/or hydrolysis to the parent 5'-monophosphate prior cell-membrane penetration [87]. To overcome this limitation, a family of simple neutral triesters of cyclic AMP was prepared [86,88]; compounds were found to cross cell membranes and be able to deliver 5'-monophosphates intracellularly [86,88].

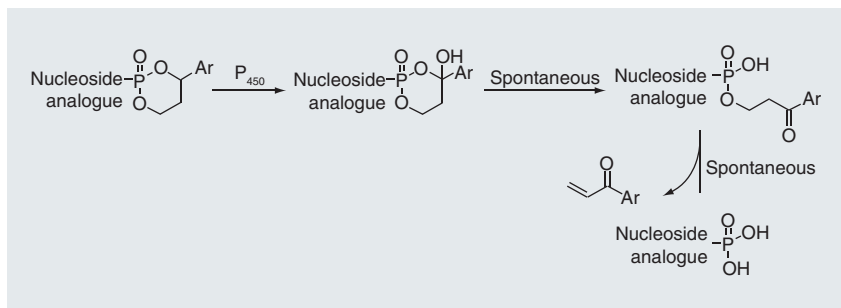


Figure 11. Metabolic activation of HepDirect prodrugs.

A simple 3',5'-cyclic triester approach was applied to anti-HCV nucleosides giving proof-of-concept by delivering 5'-monophosphates into the cells both *in vitro* and *in vivo* [89]. Other cyclic approaches including 3',5'-cyclic phosphoramidates [90] and 3',5'-cyclicSATE [83] prodrugs were also investigated and will be discussed below (FIGURE 10).

■ HepDirect prodrugs

In 2004 Erion *et al.* reported a specific prodrug strategy dependent on cytochrome P450 catalyzed cleavage, resulting in high liver-specific drug delivery [91]. The HepDirect prodrugs are substituted cyclic 1,3-propanyl esters of NA phosphates or phosphonates, designed to undergo enzyme catalyzed oxidative cleavage in the liver [91].

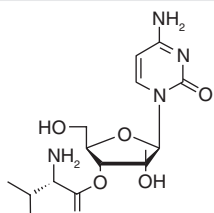
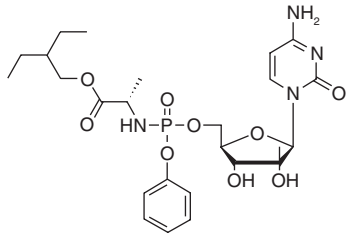
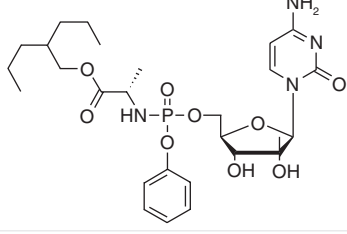
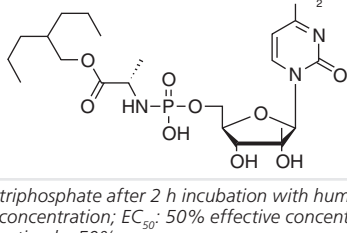
Oxidation of the benzylic carbon by CYP3A4 results in the formation of a cyclic hemiketal that undergoes spontaneous ring opening and then β -elimination to give the active monophosphorylated species and aryl-vinyl ketone by-product (FIGURE 11). The released monophosphate is further converted to the corresponding triphosphate form [91]. The formation of aryl vinyl ketone had raised toxicological concerns, due to the possible alkylation of DNA and essential proteins. It was postulated that high levels of glutathione present in the gut and liver could detoxify vinyl ketones through a rapid and quantitative 1,4-addition reaction and, thus, minimize the toxicity issues [91,92]. The HepDirect prodrug approach was successfully applied to several different NAs resulting in the discovery of pradefovir (prodrug of adefovir). This compound was advanced into clinical trials for the treatment of hepatitis B [93]. Application of the HepDirect technology to anti-HCV nucleosides will be discussed below.

ProTides of anti-HCV NAs

■ 2'-C-methylcytidine ProTides

During the development of NM283, it was noted that the triphosphate of 2'-C-methylcytidine (5) was highly active against purified viral polymerase ($IC_{50} = 0.09\text{--}0.2 \mu\text{M}$). However, the parent nucleoside was only moderately active when tested in HCV replicon cells [39]. Recent studies indicate that 2'-C-methylcytidine is a poor substrate of 2'-deoxycytidine kinase and, therefore, NM107 was not efficiently converted to its active-triphosphate form. To overcome this limitation the aryloxy phosphoramidate approach was applied to NM107. Broad investigation of different amino acid esters and aryloxy moieties

Table 2. SAR of 2'-C-methylcytidine ProTides 3 and 23–25.

| Compound | Structure | EC ₅₀ (μM) | CC ₅₀ (μM) | NTP, AUC _{0–2 h} (μM•h) [†] |
|----------|---|-----------------------|-----------------------|---|
| 3 |  | 7.6 | 100 | 16 |
| 23 |  | 0.22 | 7 | 460 |
| 24 |  | 0.04 | 2 | 107 |
| 25 |  | 8.2 | >100 | 511 |

[†]Intracellular level of triphosphate after 2 h incubation with human hepatocytes.

CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit hepatitis C virus replication by 50%.

(including substituted and unsubstituted phenols vs 1-naphthol) led to the phosphoramidate derivatives (**23–25**; **TABLE 2**) [94].

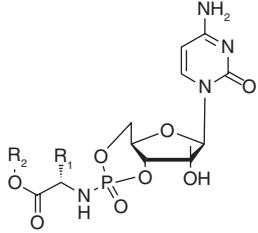
The activity of newly synthesized phosphoramidates in most of the cases was greatly improved compared with NM283. Their activation to the nucleoside triphosphate was measured in hepatocytes of several species (including humans, rats and dogs). All of the data showed that phosphoramidates could produce higher levels of 2'-C-methylcytidine triphosphate compared with **3**. Preparation of phosphoramidate monoesters bearing highly lipophilic amino acid esters (**25**) resolved the toxicity issues but with a decrease in activity at the same time (~200-fold vs the most active [24]). The most interesting compound in this series was compound **25**, which gave the highest NTP concentrations in human hepatocytes (**TABLE 2**). High liver triphosphate

levels in hamster were obtained upon subcutaneous administration, reaching an AUC_{0–48h} of 4600 μM/h, after 10 mg/kg dosing. However, when the compound was administered orally to rats, the levels of nucleoside triphosphate in the liver were below the limit of detection, suggesting a lack of oral bioavailability [94].

The 3',5'-cyclic phosphoramidate approach was another technology applied to 2'-C-methylcytidine. The rationale behind this family of compounds was a reduction in the degree of rotational freedom compared with aryloxy phosphoramidates and possible enhancement in cell uptake. Additionally, a lack of aryl unit may have a potential impact on cellular toxicity.

A family of different 3',5'-cyclic phosphoramidates was synthesized, followed by phosphate isomer separation (**TABLE 3**) [90]. All

Table 3. Antiviral activity and conversion to nucleotide triphosphate in human hepatocytes of 3',5'-cyclic phosphoramidates.

|  | | | | |
|--|-----------------|--------------------|-----------------------|--|
| Compound [†] | R ₁ | R ₂ | EC ₅₀ (μM) | NTP, AUC _{0-2h} [†] (μM•h) |
| 3 | - | - | 7.6 | 16±8 |
| 26 SE | CH ₃ | Et | 4 | 6±1 |
| 26 FE | CH ₃ | Et | >20 | 23±3 |
| 27 SE | CH ₃ | c-heptyl | >10 | 94±27 |
| 28 SE | CH ₃ | 2-(hexyloxy)-ethyl | 14 | 94±14 |
| 29 SE | CH ₃ | 2-ethyl-butyl | >20 | 3±0 |

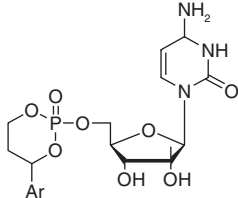
[†]Intracellular 2'-C-methylcytidine triphosphate (NTP) after incubation at 10 μM with cryopreserved human hepatocytes. CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; FE: Fast eluting diastereoisomer; SE: Slow eluting diastereoisomer.

reported, newly synthesized 3',5'-cyclic phosphoramidates of 2'-C-methylcytidine were only moderately active and did not provide improvement in antiviral potency compared with **3** or 2'-C-methylcytidine itself. Results from human hepatocytes revealed that compounds were efficiently converted to the corresponding triphosphates *in vitro* (**27** & **28**; TABLE 3). Subcutaneous administration resulted in up to 50-fold higher levels of NTP compared with triphosphate produced from 2'-C-methylcytidine, but during *in vivo* studies using the hamster animal model,

low bioavailability and/or instability in GI tract were reported [90]. No further development of this class of compounds was published.

Recently, a new family of HepDirect prodrugs of 2'-C-methylcytidine has been reported [95]. Screening of 1-aryl-1,3-propanediol derivatives led to the identification of compound **33** (TABLE 4) 3,5-difluorophenyl derivative, providing the highest levels of 2'-C-methylcytidine 5'-triphosphate in rat liver, following oral dosing across the family. Biological evaluation of single isomers of **33** revealed that the rate of NTP

Table 4. Activation of adenosine HepDirect prodrugs in rat hepatocytes and liver.

|  | | | | |
|--|-----------|----------------------------------|--|---|
| Aryl | Compound | NTP [†] (nmol/g; 25 μM) | NTP [‡] (nmol/g; 5 mg/kg [¶] i.p.) | NTP [§] (nmol/g; 10 mg/kg [¶] p.o.) |
| — | 3 | 0 | 1.9 | <1 |
| 3-F-phenyl | 30 | 226 | 50 | 1.6 |
| (S)-3-Cl-phenyl | 31 | 310 | | 4.9 |
| 3-Br-phenyl | 32 | 148 | 149 | 7.5 |
| 3,5-F ₂ -phenyl | 33 | 134 | 192 | 15.1 |
| 3,5-Cl ₂ -phenyl | 34 | 168 | 71 | 1.9 |
| 2,3-F ₂ -phenyl | 35 | 71 | 2.5 | <1 |

[†]Activation of adenosine HepDirect prodrugs in rat hepatocytes (25 μM, 2 h).
[‡]Activation of adenosine HepDirect prodrugs in rat liver (at 1 or 3 h following intraperitoneal dosing).
[§]Activation of adenosine HepDirect prodrugs in rat liver (at 3 or 5 h following oral dosing).
[¶]Nucleoside equivalents, dose normalized to 5 mg/kg i.p. and 10 mg/kg p.o.
NTP: Nucleoside triphosphate.

formation from the S-isomer was approximately fourfold greater than that from the R-isomer (based on the AUC of NTP in the liver). Oral and iv. administration of single S-isomer to rhesus macaques in a multiple-dose study showed very good plasma exposure following iv. dosing comparing to the poor oral bioavailability. Further evaluation of efficacy in two HCV-infected chimpanzees revealed that oral administration of 33 S-isomer (10 mg/kg/day for 7 days) resulted in 1.3–1.5 log IU/ml reduction in viral RNA levels, compared with 3.6–4.8 log IU/ml from iv. dosage of 4 mg/kg/day for 5 days. The data indicate that the poor antiviral efficacy of 30-S isomer following oral administration is likely due to the limited oral bioavailability of this class of molecules [95].

■ β -D-2'-deoxy-2'-fluoro-2'-C-methyluridine ProTides

As already mentioned during metabolic studies of PSI-6130 in human hepatocytes, cytidine analogues can undergo deamination leading to the formation of poorly active uridine metabolites, for example RO2433 (PSI-6206; **36**) [50]. Subsequent studies indicated that the triphosphate form of RO2433 was a potent inhibitor of viral NS5B polymerase with an IC_{50} value of 0.52 μ M. The mean half-lives of PSI-6130 triphosphate and RO2433 triphosphate were 4.7 and 38 h, respectively, indicating that RO2433 might have an advantage over PSI-6130 when tested *in vivo*, maintaining more constant concentrations of the triphosphate over the dosing period. Despite the potency exhibited in the enzyme assay, **36** was not active in replicon cells up to 100 μ M in concentration. Additional studies revealed that the RO2433 is a poor substrate for the deoxycytidine kinase and, therefore, the formation of RO2433-monophosphate species in human hepatocytes was most likely accomplished by the deamination of PSI-6130-monophosphate (Figure 12) [50,96].

To take advantage of the preferable half-life of the β -D-2'-deoxy-2'-fluoro-2'-C-methyluridine triphosphate, the aryloxy phosphoramidate approach was applied to **36** in order to deliver the monophosphate intracellularly (Table 5) [97].

Study of the ester unit (where amino acid was L-alanine and phenyl was kept as aryl moiety), showed that the small simple alkyl and branched alkyl esters provide the best submicromolar activity. The SAR of the aryl unit demonstrated that 1-naphthyl provided the highest potency, however, this substitution also led to

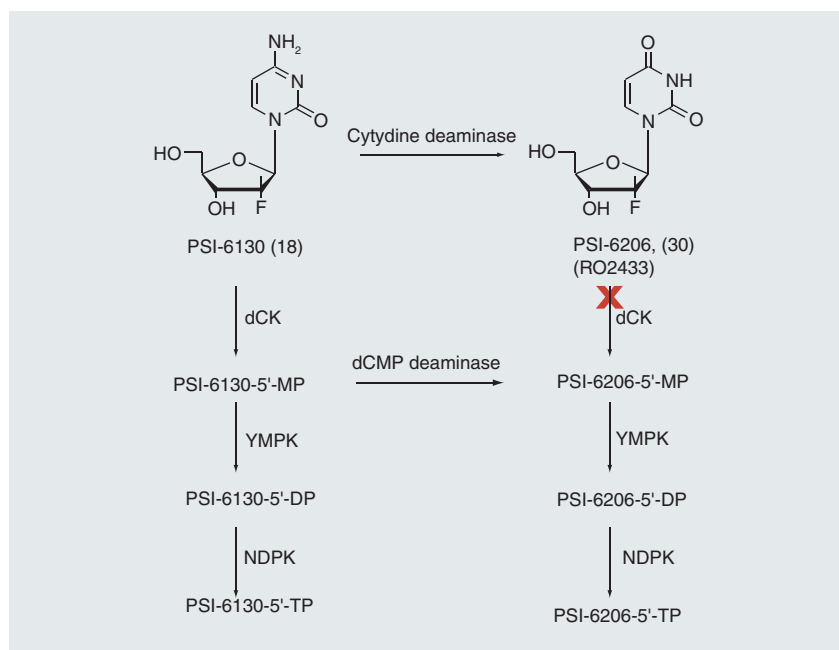


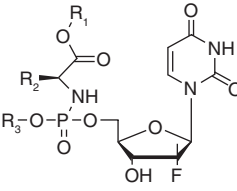
Figure 12. Phosphorylation pathway of PSI6130 and PSI6206 (RO2433).

5'-MP: 5'-monophosphate; 5'-DP: 5'-diphosphate; 5'-TP: 5'-triphosphate; dCK: Deoxycytidine kinase; dCMP: Deoxycytidylate deaminase; NDPK: Nucleoside diphosphate kinase; YMPK: Uridine/cytidine monophosphate kinase.

the significant cytotoxicity (**41**). Halogenated phenyl substitutions provided good activity with moderate toxicity. Examination of different amino acids showed that L-alanine was preferred. Extensive SAR studies around the phosphorus center resulted in the synthesis of **38**, PSI-7851 (as a 1:1 diastereomeric mixture at the phosphate), exhibiting submicromolar activity in replicon assay ($EC_{90} = 0.52 \mu$ M). The compound was shown to be stable in simulated gastric and intestinal fluids. S9 data predicted likely efficient first pass metabolism, desired for anti-HCV activity. Pharmacokinetic studies revealed that **38** can generate high levels of PSI-6206–5'-triphosphate in the liver without cellular toxicities (including mitochondrial and bone marrow toxicity) [97].

In a single ascending-dose trial in healthy volunteers, PSI-7851 was shown to be safe and well tolerated at all tested doses (25–800 mg). Forty treatment-naïve patients were enrolled for the Phase I multiple ascending-dose study. HCV RNA reduction was observed in a dose-dependant manner, with a maximum of 1.01 log IU/ml and 1.96 log IU/ml decline at day 3, for 200 mg and 400 mg daily doses, respectively. PSI-7851 was well tolerated across all tested doses with no discontinuations. No serious adverse and/or dose-dependant events were observed [98].

Table 5. Biological evaluation of β -D-2'-deoxy-2'-fluoro-2'-C-methyluridine ProTides.

|  | | | | | |
|--|---------------|---|---------|-----------------------|--|
| Compound | R1 | R2 | R3 | EC ₉₀ (μM) | Inhibition of cellular rRNA replication (%) [†] |
| 36 | | | | 3.9 | 0.0 |
| 37 | Me | CH ₃ | Ph | 0.91 | 0.0 |
| 38 | <i>i</i> -Pr | CH ₃ | Ph | 0.52 | 25.9 |
| 39 | <i>c</i> -Hex | CH ₃ | Ph | 0.25 | 61 |
| 40 | Bn | CH ₃ | Me | 0.13 | 74.3 |
| 41 | Me | CH ₃ | 4-F-Ph | 0.69 | 16.8 |
| 42 | Me | CH ₃ | 4-Cl-Ph | 0.58 | 62.8 |
| 43 | Me | CH ₃ | 1-Napth | 0.09 | 95.4 |
| 44 | Me | CH ₃ | Et | >50 | 16.8 |
| 45 | Me | H | Ph | 22.11 | 0.0 |
| 46 | Me | CH(CH ₃) ₂ | Ph | >50 | 0.0 |
| 47 | Me | CH ₂ CH(CH ₃) ₃ | Ph | 5.4 | 0.0 |
| 48 | Me | CH ₂ CH ₂ SCH ₃ | Ph | 60.13 | 24.1 |
| 49 [‡] | <i>i</i> -Pr | CH ₃ | Ph | 0.42 | ND |

[†]At 50 μM concentrations.
[‡]Sp isomer.
 EC₉₀: 90% effective concentration or compound concentration required to inhibit HCV replication by 90%;
 ND: Not determined.

Alongside clinical development of PSI-7851, further work on separated isomers was carried out. In clone A replicon assay, EC₉₀ values of 7.5 and 0.42 μM (for Rp and Sp [49] isomers, respectively) were reported. During the evaluation of intracellular 5'-triphosphate production (in human hepatocytes), the Sp isomer was found to produce approximately 14% higher concentration of triphosphate than the Rp isomer. Both isomers were found to be nontoxic in different cell lines up to 100 μM concentration [97,99]. As a result, the single Sp isomer of PSI-7851 (PSI-7977) progressed into Phase IIa clinical study, where HCV treatment-naïve patients were dosed with 100, 200 or 400 mg q.d. of PSI-7977 in combination with SOC, for 28 days. Patients achieved an approximate average mean of 5.3 log IU/ml decline in viral RNA, however dose-dependent activity was not observed. RVR in 88–93% of infected patients was achieved. There were no serious adverse events reported during 4-weeks treatment. All reported adverse events were mild to moderate, with fatigue, nausea and joint pains being the most common. Following the positive Phase IIa clinical results, a Phase IIb was initiated in which PSI-7977 at doses of 200 and 400 mg q.d., was combined

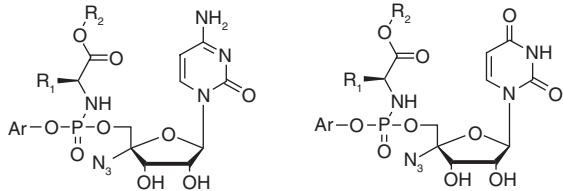
with SOC for 12 weeks, as a treatment regimen for patients infected with genotype 1, 2 and 3 [100–103,303,304].

PSI-7977 is also being studied in combination with protease inhibitor BMS-790052 and another polymerase inhibitor PSI-938 in Phase I clinical trials [101,305]. Recently, Pharmasset entered into a clinical collaboration agreement with Tibotec Pharmaceuticals to evaluate in a Phase II study the safety and efficacy of PSI-7977 in combination with protease inhibitor TMC435, during 12-week all oral, q.d., and interferon-free regimen in patients infected with genotype 1 HCV and PEG-IFN/RBV nonresponding patients [306].

■ 4'-C-azidocytidine & 4'-C-azidouridine ProTides

As previously mentioned, 4'-C-azidocytidine was found to inhibit HCV replication in a low micromolar range, when tested in replicon assay. The corresponding 5'-triphosphate (**15**) inhibits NS5B polymerase in a low nanomolar range in enzyme-based assay. Interestingly, the 4'-C-azidouridine analogue **7** did not exhibit any anti-HCV activity in cell-based systems, while its triphosphate was found to be an efficient

Table 6. Biological evaluation of 4'-C-azidocytidine and 4'-C-azidouridine ProTides.



| Compound | Base | Aryl (Ar) | R ₁ | R ₂ | EC ₅₀ (μM) | CC ₅₀ (μM) |
|-----------|------|-----------------|---|----------------|-----------------------|-----------------------|
| 6 | C | - | - | - | 1.28 | ND |
| 50 | C | Ph | CH ₃ | Bn | 6.0 | >100 |
| 51 | C | Ph | CH ₃ | Et | 0.38 | >100 |
| 52 | C | Ph | CH ₃ | <i>i</i> -Pr | 0.99 | >100 |
| 53 | C | <i>p</i> -Me-Ph | CH ₃ | Bn | 0.51 | >100 |
| 54 | C | <i>p</i> -Me-Ph | CH ₃ | Et | 2.9 | >100 |
| 7 | U | - | - | - | >100 | >100 |
| 55 | U | Ph | CH ₃ | Et | 1.3 | >100 |
| 56 | U | Ph | CH ₃ | <i>i</i> -Pr | 0.77 | >100 |
| 57 | U | Ph | CH ₃ | Bn | 0.61 | >100 |
| 58 | U | Ph | H | Bn | 1.6 | >100 |
| 59 | U | Ph | CH ₂ CH(CH ₃) ₂ | Bn | 2.3 | >100 |
| 60 | U | Ph | CH(CH ₃) ₂ | Bn | <100 | >100 |
| 61 | U | Napth | CH ₃ | Bn | 0.22 | >100 |

CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%.

inhibitor of HCV RdRp in an enzyme assay, with an IC₅₀ of 0.22 μM [45]. The lack of activity in the replicon assay was associated with poor initial phosphorylation of **7** and, therefore, the phosphoramidate approach was investigated as a tool for monophosphate delivery of both **6** and **7**. Extensive investigation around the phosphate center including different amino acid esters and aryl moieties provided novel compounds with moderate (for ProTides of 4'-C-azidocytidine) and highly increased (for 4'-C-azidouridine) activity (**TABLE 6**) [102,103].

For the family of 4'-C-azidocytidine ProTides, the most active compound (**53**) exhibited three-fold improvement in potency compared with the parent nucleoside (**6**). Most of the reported compounds have either slightly improved or retained the potency of **6**, suggesting that the first phosphorylation of this particular NA is not an issue and the phosphoramidate approach cannot greatly improve the potency of parent nucleoside. By contrast, in the case of 4'-C-azidouridine the phosphoramidate approach greatly enhanced anti-HCV activity *in vitro* (>450-fold for **61**), without apparent toxicity up to 100 μM; successfully bypassing the rate-limiting first phosphorylation step [102,103]. No additional data on further biological evaluation of 4'-C-azidouridine phosphoramidates have since been reported.

■ 2'-C-methyladenosine ProTides

2'-C-methyladenosine (**62**) was found to be a potent inhibitor of viral replication with EC₅₀ = 0.26 μM and the corresponding adenosine 5'-triphosphate (**63**) inhibited HCV RNA polymerase at 1.9 μM (**FIGURE 13**) [34,104].

2'-C-methyladenosine was found to be a substrate for adenosine deaminase, adenylate deaminase and purine nucleoside phosphorylase, resulting in low metabolic stability and low oral bioavailability. The aryl phosphoramidate approach was applied to **58** to overcome these limitations (**TABLE 7**) [105].

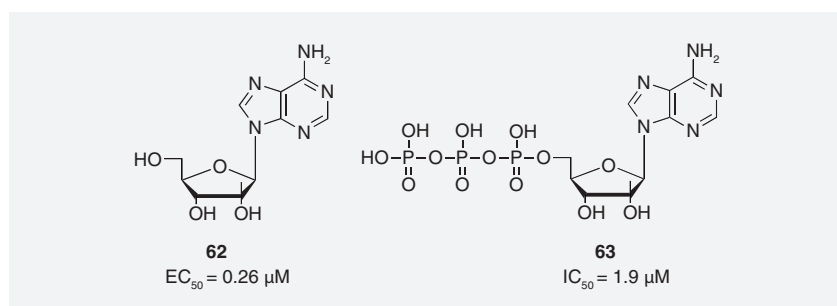
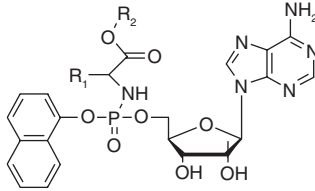


Figure 13. Structures and anti-hepatitis C virus activity of 2'-C-methyladenosine and the corresponding triphosphate.

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC₅₀: 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

Table 7. Biological activity of 2'-C-methyladenosine and corresponding phosphoramidates in hepatitis C virus Huh 5-2 replicon assay.

|  | | | | |
|--|-----------------|----------------|-----------------------|-----------------------|
| Compound | R ₁ | R ₂ | EC ₅₀ (μM) | CC ₅₀ (μM) |
| 62 | | | 0.25 | >50 |
| 64 | CH ₃ | Et | 0.24 | >50 |
| 65 | CH ₃ | Bn | 0.27 | >50 |
| 66 | CH ₃ | <i>t</i> -Bu | 4.18 | >50 |

CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%.

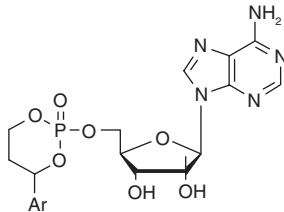
Application of phosphoramidate technology to **61** did not improve its potency, although **64** and **65** showed similar activity to the parent nucleoside (**62**) [105]. Further development of this class of phosphoramidates of 2'-C-methyladenosine was not continued.

The HepDirect approach was also applied to 2'-C-methyladenosine in order to deliver the 5'-monophosphate into the cells. It was expected that preparation of 1-aryl-1,3-propanyl prodrugs would improve the metabolic stability of the parent nucleoside, especially in terms of deamination and, as a result, therapeutic levels of 2'-C-methyladenosine triphosphate would be achieved in the liver after oral administration.

A SAR study of aryl moieties was evaluated in rat hepatocytes at 25 μM concentration (**TABLE 8**) [106].

Data indicate that the HepDirect approach was successful in delivering high levels of 2'-C-methyladenosine triphosphates when tested in rat hepatocytes *in vitro* (**67–73**; **TABLE 7**; NTPα). In general, different halogenated phenols and pyridyl substitutions were well tolerated and efficiently oxidized yielding the desired monophosphate, which was subsequently phosphorylated to the active species. During *in vivo* evaluations, substantial levels of liver triphosphate were achieved (**67–73**; **TABLE 8**; NTPβ), where compounds were iv. administered to rats.

Table 8. Activation of adenosine HepDirect prodrugs.

|  | | | | | | |
|---|-----------|---------------------------|---------------------------|-----------------------------------|---------------------------|---------------------------|
| Aryl (Ar) | HepDirect | | | HepDirect 2',3'-carbonate prodrug | | |
| | Compound | NTP [†] (nmol/g) | NTP [‡] (nmol/g) | Compound | NTP [†] (nmol/g) | NTP [‡] (nmol/g) |
| | 62 | 209 | - | 62 | 209 | - |
| (S)-3-Cl-phenyl | 67 | 389 | 2.9 | 74 | 298 | 29.2 |
| 3,4-Cl ₂ -phenyl | 68 | 396 | 2.0 | 75 | 97 | 11.8 |
| 3,5-Cl ₂ -phenyl | 69 | 585 | 2.4 | 76 | 49 | 5.0 |
| 3-F-phenyl | 70 | 328 | 4.1 | 77 | 313 | 22.9 |
| 2,3-F ₂ -phenyl | 71 | 323 | 8.2 | 78 | 280 | 14.7 |
| 3,5-F ₂ -phenyl | 72 | 139 | 4.1 | 79 | 113 | 11.2 |
| (S)-4-pyridyl | 73 | 47 | 3.6 | 80 | 185 | 28.3 |

[†]Activation of adenosine HepDirect prodrugs in rat hepatocytes (25 μM, 2 h).
[‡]Activation of adenosine HepDirect prodrugs in rat liver (at 3 h following oral dosing, 50 mg/kg).
NTP: Nucleoside triphosphate.

However, oral administration to rats resulted in a greater than fivefold lower level of liver triphosphate (based on the AUC of NTP in liver) and bioavailability was found to be less than 5%. To overcome bioavailability issues, modifications of the *N*-6 amino and 2',3'-hydroxyl groups were investigated, resulting in compounds (**74–80**; **TABLE 8**). The 2',3'-carbonates afforded approximately tenfold increases in liver triphosphate levels (after oral administration to rats) compared with simple HepDirect ProTides [106]. No additional data suggesting further development of this class of 2'-*C*-methyladenosine ProTides have been published.

■ 7-deaza-2'-*C*-methyladenosine ProTides

Modifications of the adenine base of 2'-*C*-methyl analogue were considered in order to increase metabolic stability of **62**. The 7-deaza-2'-*C*-methyladenosine derivative, MK0608 (**81**; $EC_{50} = 0.25 \mu\text{M}$; **FIGURE 14**) was found to be effective against HCV replication and, furthermore, the compound was not a substrate for adenosine deaminase or purine nucleoside phosphorylase [107].

The compound showed efficacy in genotype 1a and 3a infections and reached late pre-clinical development. Two chimpanzees were dosed orally with MK0608 (1 mg/kg, per day) for 37 days. This led to a 3^{-5} log reduction in serum HCV RNA levels. After dosing, viral rebound occurred in both treated chimpanzees. Despite this MK0608 has never progressed into clinical trials [107].

■ 2'-*C*-methylguanosine ProTides

Another member of the 2'-*C*-methyl purine nucleoside family: 2'-*C*-methylguanosine (**82**) has also been shown to be a modest anti-HCV agent, with an EC_{50} value of $3.5 \mu\text{M}$. The triphosphate of 2'-*C*-methylguanosine showed $IC_{50} = 0.13 \mu\text{M}$ (**FIGURE 15**) [104]. Replicon activity of **82** was lower than expected from the intrinsic polymerase inhibition of the triphosphate species (**83**). Insufficient phosphorylation of **82** was suggested to limit the antiviral potential of 2'-*C*-methylguanosine [105], therefore two different ProTide approaches were applied to **83**, resulting in two compounds advanced into clinical trials

■ IDX184

IDX184 is a first example of a 2'-*C*-methylguanosine monophosphate pro-drug advanced into clinical trials. IDX184 (**80**)

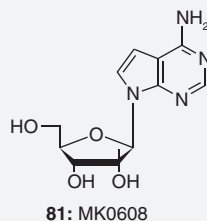


Figure 14. 7-deaza-2'-*C*-methyladenosine.

is a hybrid SATE prodrug (**FIGURE 16**) in which two different groups, SATE moiety and benzylamine, mask the 5'-monophosphate [84]. The complete metabolic pathway of IDX184 is not yet fully disclosed. *In vitro* mechanistic studies indicate that the release of 5'-monophosphate relays on cytochrome-450 dependent and independent processes [84]. IDX184 was shown to be a potent inhibitor of viral replication in replicon assay $EC_{50} = 0.4 \mu\text{M}$ without any associated toxicity ($CC_{50} > 100 \mu\text{M}$) [108].

Preclinical combinatorial studies with SOC and protease inhibitor IDX320 resulted in an additive effect. Evaluation in animal models demonstrated the proof-of-concept that IDX184 can produce high levels of intracellular 2'-*C*-methylguanosine 5'-triphosphate compared with the parent nucleoside when administered orally [109,110]. In a subsequent study, a 2.3 log IU/ml decline in viraemia was achieved in HCV-infected chimpanzees at a 10 mg/kg daily dose over a 3-day treatment at days 3 and 4. IDX184 was advanced into clinical trials [110]. The aim of the Phase I study was to evaluate safety, tolerability and pharmacokinetics of single doses of IDX184 in healthy volunteers. A total of 48 healthy volunteers were enrolled and divided into six groups of eight subjects; six active and two placebo recipients.

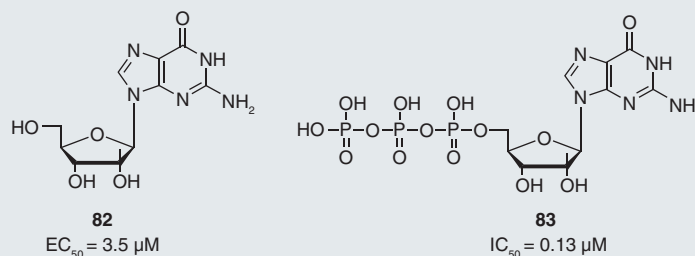


Figure 15. Structures and anti-HCV activity of 2'-*C*-methylguanosine and corresponding triphosphate.

EC_{50} : 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC_{50} : 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

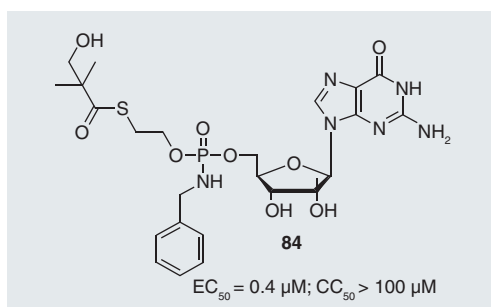


Figure 16. Structure and anti-hepatitis C virus activity of IDX184.

EC_{50} : 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC_{50} : 50% cytotoxic concentration.

During the trial, single doses of 5–100 mg (q.d., for 3 days) were generally well-tolerated; no serious adverse events or dose-dependent toxicities were observed. The highest decline of HCV RNA was observed for the 100 mg dose = 0.74 log IU/ml. Pharmacokinetic analyses indicate liver targeting, with very low plasma concentrations of 2'-C-methylguanosine and IDX184. The oral bioavailability of IDX184 remains unknown, although $\geq 20\%$ is suggested [84]. Successful results of the Phase I trial led to the initiation of Phase II trials. Phase IIa was a randomized, double blind, placebo-controlled and sequential dose-escalation study. The objective was to evaluate safety, tolerability, pharmacokinetics and antiviral activity of IDX184 in combination with PEG-IFN/RBV in treatment-naïve HCV genotype 1 infected patients over 14 days. Patients received a daily dose of 50–200 mg IDX184 per day. In the 100 and 200 mg cohorts, b.i.d. and q.d. regimens were compared. A 2.7–4.1 log IU/ml dose-dependent reduction of viral load was observed at day 14. No serious adverse events related to the IDX184 were observed [111,112]. Additional Phase I combination therapy with NS3 protease inhibitor, IDX320, was evaluated in healthy volunteers. Safety data showed three cases of serious adverse events (liver function abnormalities) during a drug–drug interaction study and the program was placed on full clinical hold, awaiting resolution of the adverse events [307]. At the beginning of 2011, the US FDA removed full clinical hold for IDX184 and the program was placed on partial hold. The full toxicological data revealed that the toxicity was most likely caused by the IDX320. The Phase IIb trial of IDX184 in combination with PEG-IFN and

RBV under a partial clinical hold was initiated in July 2011 [308].

■ INX-08189

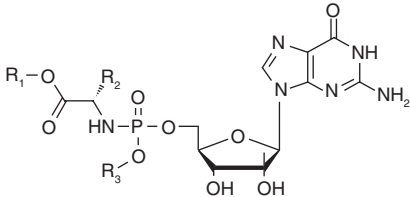
INX-08189 represents the second example of a 2'-C-methylguanosine 5'-monophosphate prodrug, also advanced into clinical trials. This compound is an aryloxy phosphoramidate double prodrug of 2'-C-methylguanosine. The development of INX-08189 is based on an extensive study of different ProTides of 2'-C-methylguanosine, including variation of ester, amino acid and aryl moieties (Table 9) [105,113].

The initial series of compounds was found to be 10–30-fold more potent than the parent nucleoside without any increase in cellular toxicity (85–95; Table 9). Satisfactory plasma stability across different species (mouse, rat, dog and human) was observed. *In vitro* liver and intestinal S9 data supports the notion of liver targeting by this class of molecules [113]. Further investigation of this family led to the modifications of the base unit in order to increase lipophilicity of these ProTides and, therefore, improve their cellular uptake and pharmacokinetic properties. Substitutions at the C-6 position led to the identification of the 6-O-methyl derivative and, subsequently, INX-08189 (97; Table 10) [114,115].

The monophosphate functionality of INX-08189 is masked by α -naphthyl and 2,2-dimethylpropoxy-L-alanyl moieties. In addition to the general phosphoramidate activation pathway, metabolic conversion of the 6-O-methyl substitution of the purine base also takes place in INX-08189 [116]. Metabolic studies confirmed that this conversion is accomplished at the monophosphate level. INX-08189 exhibits nanomolar activity in HCV 1b replicon assay ($EC_{50} = 0.01 \mu M$ and $CC_{50} = 7 \mu M$) and was found to be active against replicon 1a and 2a with EC_{50} values of 0.012 μM and 0.0009 μM , respectively. Supported by preclinical data INX-08189 was advanced into clinical trials as a mixture of phosphate diastereoisomers. No difference in antiviral potency of the individual isomers was found when tested in replicon assay [115–117].

Some 42 healthy volunteers were enrolled for the trial. In a single ascending dose, placebo controlled study, recipients were administered with doses ranging from 3 to 100 mg. INX-08189 was generally well tolerated at all doses, with no drug-related, serious adverse events. No

Table 9. SAR of 2'-C-methylguanosine phosphoramidates.



| Compound | R ₁ | R ₂ | R ₃ | EC ₅₀ (μM) | CC ₅₀ (μM) | Parent remaining (%) [†] |
|-----------|----------------|---|----------------|-----------------------|-----------------------|-----------------------------------|
| 82 | - | - | - | 3.5 | >100 | ND |
| 85 | Me | CH ₃ | 1-Naph | 0.21 | >50 | 2 |
| 86 | Neopentyl | CH ₃ | 1-Naph | 0.06 | >100 | 1 |
| 87 | <i>i</i> -Pr | CH ₃ | 1-Naph | 0.17 | >100 | 14 |
| 88 | <i>c</i> -Hex | CH ₃ | 1-Naph | 0.045 | >100 | 3 |
| 89 | Bn | CH ₃ | 1-Naph | 0.062 | >100 | 1 |
| 90 | Bn | CH ₃ | 1-Naph | 0.76 | >100 | 89 |
| 91 | Bn | CH ₃ | 1-Naph | 0.12 | >50 | 2 |
| 92 | Bn | CH(CH ₃)CH ₂ CH ₃ | 1-Naph | 0.9 | >100 | 100 |
| 93 | <i>i</i> -Pr | CH ₂ CH ₂ SCH ₃ | 1-Naph | 0.34 | >100 | 76 |
| 94 | Bn | CH ₃ | 2-Naph | 0.17 | >50 | 1 |
| 95 | Bn | CH(CH ₃) ₂ | 2-Naph | 1.7 | >100 | 99 |

[†]Percentage remaining in mouse plasma (at 2–4°C, during 30 min incubation, final concentration of 1 μg/ml in 1 ml of plasma).
 CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%.

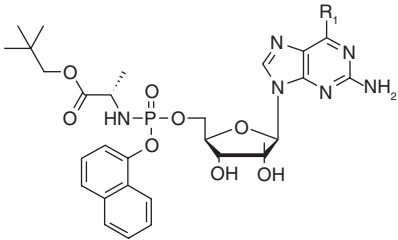
dose-related trends were observed and pharmacokinetic data support once a day, oral dosing of INX-08189. The compound was progressed into Phase Ib trials and the trial carried out on 70 HCV genotype treatment-naïve patients, receiving an all-oral regimen of INX-08189 and RBV against placebo-controlled group. Patients were dosed once daily at 9–100 mg. INX-08189 demonstrated dose-dependent antiviral activity with a decline in HCV RNA of 1.00 log IU/ml for dosage as low as 25 mg at day 7 when tested alone, and 1.56 log IU/ml when tested with RBV double regimen. Additional data from the Phase Ib clinical studies indicated that INX-08189 was generally well tolerated at all tested doses. There were no significant adverse events associated with the drug, other reported adverse events were mild or moderate, with headache being the most common. There were no discontinuations of treatment due to adverse events. INX-08189 entered Phase II clinical trials in August 2011 [118,309].

■ 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine ProTides

Investigations of 2'-deoxy-2'-α-fluoro-2'-C-methylribofuranose have been successful in delivering potent and selective anti-HCV pyrimidine nucleosides (NM283, R1626 or RG7128).

As a part of continuing effort to discover new and potent inhibitors of HCV replication, 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine (**101**) was investigated. The compound exhibited only modest activity in replicon assay (EC₅₀ = 69.2 μM) but its triphosphate demonstrated good activity against viral polymerase (**102**; IC₅₀ = 5.94 μM; **FIGURE 17**) [119].

Table 10. SAR of 6-modified 2'-C-methylguanosine phosphoramidates.



| Compound | R ₁ | EC ₅₀ (μM) | CC ₅₀ (μM) |
|------------|----------------|-----------------------|-----------------------|
| 86 | OH | 0.06 | >100 |
| 96 | Cl | 0.012 | 16 |
| 97 | OMe | 0.01 | 7 |
| 98 | OEt | 0.01 | 10 |
| 99 | NHMe | 0.035 | 49 |
| 100 | SMe | 0.037 | 41 |

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; CC₅₀: 50% cytotoxic concentration.

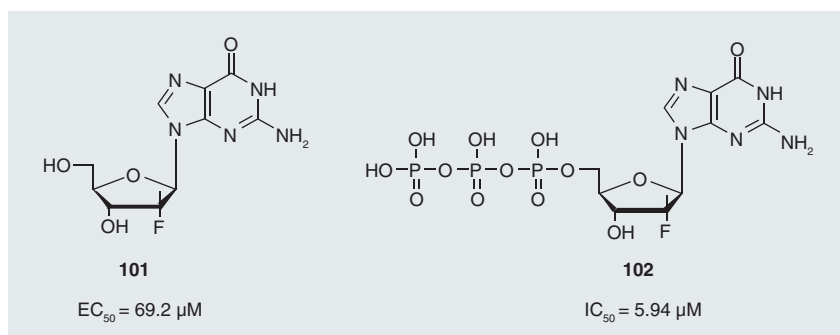


Figure 17. Structures and anti-HCV activity of 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine and corresponding triphosphate.

EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; IC₅₀: 50% compound concentration required to inhibit activity of HCV polymerase by 50%.

■ PSI-938

Several 6-modifications significantly improved antiviral activity of **101**. A 3',5'-cyclic ProTide strategy was applied to the latter. It was shown, that 3',5'-cyclic prodrugs of 6-substituted purine nucleosides are metabolized into the 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine triphosphate. Extensive studies of different simple alkyl and aryl esters combined with different modifications in the 6-position showed that both alkoxy and alkyl amine substituents are accepted at C-6 position, while methyl was found to be the best as a cyclic phosphate masking moiety (**TABLE II**) [120].

Table 11. SAR of 3',5'-cyclic prodrugs of 6-substituted 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine.

| Compound | R ₁ | R ₂ | EC ₉₀ (μM) | CC ₅₀ (μM) |
|-----------------|----------------|----------------|-----------------------|-----------------------|
| 103 | OH | Me | 11.75 | >65 |
| 104 | OEt | Me | 0.41 | >30 |
| 105 | OPr | Me | 0.83 | >30 |
| 106 | OEt | Me | 0.01 | 10 |
| 107 | NHPr | Me | 0.92 | >30 |
| 108 | OEt | <i>n</i> -Pr | 13.27 | ND |
| 109 , Rp | OEt | <i>i</i> -Pr | 1.37 | >100 |
| 109 , Sp | OEt | <i>i</i> -Pr | 0.69 | >100 |
| 110 , Rp | OPr | <i>n</i> -Pr | 23.83 | ND |
| 110 , Sp | OPr | <i>n</i> -Pr | 5.27 | >11 |

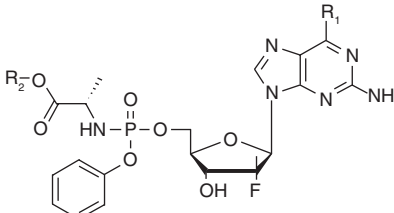
CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%; ND: Not determined..

Biological evaluation led to the identification of PSI-352938 (**109**; Rp isomer) as a potent inhibitor of HCV replication in replicon assay (EC₉₀ = 1.37 μM) without any cytotoxicity (EC₉₀ = 1.37 μM) across different cell lines (including mitochondrial and bone marrow toxicity) up to 100 μM. PSI-352938 was found to be active against all six genotypes. *In vitro* studies with PEG-IFN and RBV demonstrated that **109** could be combined to produce an additive or synergistic antiviral effect. Studies in animal models revealed production of high levels of 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine triphosphate in rat liver, after oral administration of 50 mg/kg. The metabolic pathway of **109** is not yet fully disclosed [120].

PSI-352938 was advanced into Phase I clinical trials to evaluate its safety profile, tolerability and pharmacokinetics in healthy subjects. The compound was administered once daily, ranging from 100–800 mg against placebo. The compound was generally well tolerated at all tested doses. No serious adverse events or discontinuations were recorded. No dose-related or dose-limiting adverse events/toxicities were observed, with pharmacokinetic analysis supporting q.d. dosing. PSI-352938 was progressed into Phase Ib clinical trials in 40 treatment-naive HCV genotype 1 patients. In a blind, randomized and placebo controlled study, **109** was administered at doses of 100–300 mg q.d. and 100 mg b.i.d. over 7 days. At day 8, a mean HCV RNA change from baseline of 4.31 log IU/ml, 4.64 log IU/ml, 3.94 log IU/ml and 4.59 log IU/ml in patients receiving 100 mg q.d., 200 mg q.d., 300 mg q.d. and 100 mg b.i.d., respectively, was found. 11 out of 16 patients from the 200–300 mg groups achieved RVR. Pharmacokinetic parameters were similar to the ones reported for the healthy subjects, providing good systemic exposure and further supporting once-daily dosing. PSI-938 was generally welltolerated at all tested doses. There were no reported adverse events or discontinuations over the study [121,310].

■ PSI-353661

As an outcome of the positive results of PSI-938, further development of the 6-modified 2'-deoxy-2'-α-fluoro-2'-C-methylguanosine phosphoramidates was carried out (**TABLE 12**). Synthesized phosphoramidates were shown to possess very good anti-HCV activity in HCV clone A replicon assay, without significant toxicity in different cell lines [119,122].

Table 12. SAR of 6-modified 2'-deoxy-2'- α -fluoro-2'-C-methylguanosine phosphoramidates.


| Compound | R ₁ | R ₂ | EC ₅₀ (μ M) | CC ₅₀ (μ M) |
|------------|----------------------------------|----------------|-----------------------------|-----------------------------|
| 101 | OH | - | 69.2 | >100 |
| 111 | OH | <i>i</i> -Pr | 0.75 | >100 |
| 112 | OMe | Me | 0.008 | >100 |
| 113 | OMe | <i>i</i> -Pr | 0.02 | >100 |
| 114 | OEt | Me | 0.016 | >100 |
| 115 | OEt | <i>i</i> -Pr | 0.02 | 59 |
| 116 | OPr | Me | 0.015 | 39 |
| 117 | OPr | <i>i</i> -Pr | 0.033 | 49 |
| 118 | N(CH ₂) ₃ | Me | 0.09 | >100 |
| 119 | N(CH ₂) ₃ | <i>i</i> -Pr | 0.019 | 53 |

CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%.

After comprehensive evaluation of metabolic stability and cellular triphosphate formation, PSI-353661 (single Sp isomer of **113**) was identified as a clinical candidate for the treatment of HCV infection. Extensive biological evaluations demonstrate lack of cellular and mitochondrial toxicity up to 100 μ M, without significant toxicity towards bone marrow progenitor cells (45 μ M). Cross-resistance study showed that PSI-353661 retains activity against different cell lines conferring resistance to NS5B inhibitors (both NI and NNIs). During *in vitro* studies with PEG-IFN and RBV, PSI-353661 demonstrated promising synergistic or additive antiviral effect. PSI-353661 is awaiting the start of clinical evaluation [119,122].

■ 6-Hydrazino-methylsulfonyl 2'-C-methylpurine

6-Hydrazinopurine-methylsulfonyl 2'-C-methyl nucleosides, **120** and **121**, were essentially inactive against HCV with no observed cytotoxicity (**FIGURE 18**) [83].

Compounds were not good substrates for adenosine kinase and a lack of phosphorylation to the corresponding 5'-monophosphates was postulated. To by-pass the first phosphorylation step and deliver masked 5'-monophosphates into the cells, SATE technology was applied to both NAs resulting in significant improvement in replicon potency (**TABLE 13**) [83,123].

For the most active prodrug (**127**), the potency enhancement was higher than 11,000-fold. It was demonstrated that the antiviral efficacy of these compounds was correlated with their lipophilicity, probably due to the increased permeability through the cell membrane. The stability of **127** was checked in plasma from different species. During 1 h incubation in rat plasma only 4% of **127** were found. The major detected metabolite was the cyclic-monophosphate (59%), whereas the parent nucleoside **121** was found in 18% and its monophosphate in only 19%. Compound **127** was found to be stable in monkey and human plasma during 1 h incubation period. It was suggested that

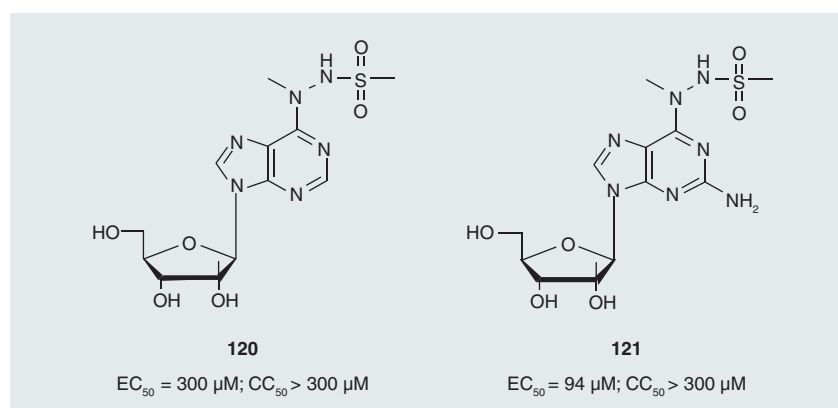
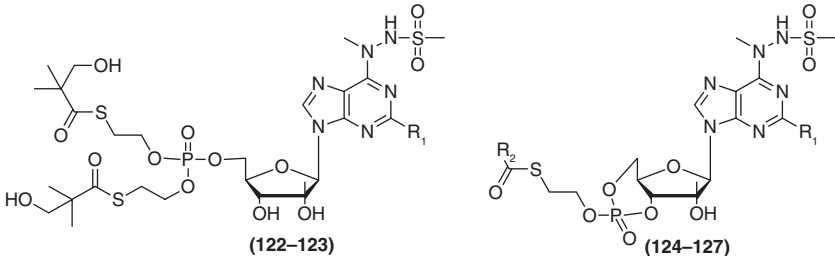




**Figure 18. Anti-hepatitis C virus activity of 6-hydrazinopurine-methylsulfonyl 2'-C-methyl nucleosides.**

Table 13. Biological evaluation of S-acetyl-2-thioethyl prodrugs of 6-hydrazino-methylsulfonyl 2'-C-methylpurines.

| | | | | |
|--|----------------------|---|-----------------------------|-----------------------------|
|  | | | | |
| Compound | R₁ | R₂ | EC₅₀ (μM) | CC₅₀ (μM) |
| I20 | H | - | 300 | 300 |
| I21 | NH ₂ | - | 94 | 300 |
| I22 | H | - | 0.06 | ND |
| I23 | NH ₂ | - | 0.024 | ND |
| I24 | H |  | 0.145 | >50 |
| I25 | NH ₂ |  | 0.022 | >50 |
| I26 | H |  | 0.039 | >50 |
| I27 | NH ₂ |  | 0.008 | >50 |

CC₅₀: 50% cytotoxic concentration; EC₅₀: 50% effective concentration or compound concentration required to inhibit HCV replication by 50%.

compounds may be able to reach hepatocytes after iv. administration [83,123]. No further data regarding development of these compounds have been reported.

Antiviral resistance for nucleoside inhibitors of RdRp

The development of resistance is dependent on a number of factors. Single nucleotide mutations are more common to occur than those requiring multiple nucleotide changes [124]. As noted earlier, the HCV virus can produce high rates of spontaneous mutations each day and, therefore, a high-resistance profile to other direct-acting antivirals should be expected [10]. The comparison between NIs and NNIs suggests that mutations in the allosteric site are more common than those in the active site. It is presumed that only a limited range of amino acid substitutions in the catalytic site would be accepted in order to maintain catalytic-site function and replication fitness. These functional restrictions can result in low levels of resistant mutants within the nontreated patients (in the absence of a drug selective pressure) [125,126].

There are several reported key resistant mutations for the clinical stage anti-HCV nucleosides. For the 4'-C-azido nucleosides (**6** & **7**), the most important isolated resistant mutants

are S96T and N142T, and for the 2'-C-methyl substituted nucleosides (**4**, **18**, **58**, **77** or **79**), this is the S282T mutant. It has been reported that all of these mutations have been associated with decreased replication fitness *in vitro*. However, it is unknown whether these primary mutants can develop compensatory mutations, which could restore deficiencies in replication fitness [127]. The 2'-C-methyl related resistant mutations lack cross-resistance with 4'-C-azido analogues [128]. However, with the discontinuation of the clinical development of 4'-C-azido analogues, all candidates that are still in development possess 2'-C-methyl substitutions. Thus far, reported *in vitro* and *in vivo* data for the 2'-C-methyl modified clinical candidates are showing high barrier to resistance for this class of nucleosides [43,116,128–130]. Nevertheless, a lack of variety in the structure of potential clinical candidates may result in the emergence of S282T slow-to-clear HCV strains or 'breakthrough' strains in treated patients.

Future perspective

We have presented an overview of the current development status of the anti-HCV nucleoside and nucleotide prodrugs. Anti-HCV NAs that are designed to specifically target viral NS5B polymerase each present different clinical

advantages and challenges. Optimization of the NA is highly challenging and multifactorial. There has to be a balance between the ability of the modified nucleoside to interact with the multiple cellular enzymes required for phosphorylation to the active triphosphate, whilst retaining metabolic stability and delivery. Furthermore, modified nucleoside triphosphates have to possess high selectivity towards viral RdRp without affinity to human polymerases.

One of the challenges associated with NAs is that their triphosphate form has to compete with relatively high concentrations of naturally occurring nucleoside triphosphates [131] and, therefore, elevated concentrations of the therapeutic nucleosides may need to be used to compensate for this unfavorable ratio.

The most significant advantage of NAs over NNIs is high potency across all six genotypes (pan-genotype coverage), whereas protease inhibitors may be limited by genotype [26]. In addition, a high genetic barrier to resistance has been reported for HCV NAs compared with the likely low genetic barriers to resistance for protease inhibitors, and particularly non-nucleoside polymerase inhibitors [115,117]. Future trends in the field of nucleoside prodrugs may include new structural classes of nucleosides that could provide better treatment options, especially for

resistant strains, exhibiting increased potency and reduced toxicity with consequent reduction of the daily dose and treatment duration. The success of directly acting anti-HCV agents will also depend on their ability to prevent the emergence of escape mutants. The evaluation of efficient nucleotide prodrugs is essential to this progress.

It is most likely that HCV eradication will be achieved not by monotherapy but by the combinations of drugs from different mechanistic classes, attacking various viral and possibly host factors, with non-cross-resistance patterns. It is also possible that two different purine and pyrimidine nucleosides/nucleotides, possessing different resistance profiles, may be used for the treatment of pre-existing resistant variants.

Although most of the current clinical trials involve addition of PEG-IFN and/or RBV to the treatment regimens, the goal of the second-generation therapy is to shift away interferon-based regimens to use exclusively direct acting antiviral agents. The future of hepatitis C therapy will probably involve personalized treatment regimens, with duration time based on viral load at the baseline, liver stage, specific enzyme levels and other parameters.

It seems likely that nucleotide prodrugs will form a key element of such therapies going forward.

Executive summary

NS5B inhibitors

- Nucleoside analogues (NAs) have been found to be potent and effective inhibitors of hepatitis C virus replication both *in vitro* and *in vivo*. Furthermore, they were found to be active against all HCV genotypes with high barriers to resistance.
- It has been demonstrated that different modifications of both the sugar and base unit can be tolerated, and high antiviral potency and selectivity can be achieved.
- 2'- and 4'-modified ribonucleoside analogues have demonstrated promising antiviral potency and selectivity as anti-HCV inhibitors; however, their polar nature may be a limitation when oral administration is required.

Nucleoside ester prodrugs

- It has been shown that simple ester prodrugs of NAs can improve their bioavailability and pharmacokinetic profiles. RG7128, the *bis*-isobutyryl ester of β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine, is an example of the most advanced nucleoside-ester prodrug in clinical trials.

ProTides of anti-HCV nucleoside analogues

- The conversion to the active triphosphates for most of the NAs was found to be problematic, with the first phosphorylation being the rate-limiting step. Several different strategies have been applied to NAs in order to deliver masked monophosphates intracellularly. Due to the nature of HCV infection a ProTide that would undergo first-pass metabolism would be preferred, as it could generate high levels of active species in the target organ (liver) without unnecessary systemic exposure.
- The use of 5'-monophosphate prodrugs has greatly enhanced inhibitory activity of several NAs in replicon assays, and the levels of the active triphosphate species were found to be much higher both in cell cultures and in liver of animal models, when dosed orally.
- During clinical studies, different ProTides (IDX184, INX08189 and PSI938) demonstrated efficacy while dosed orally, with significant rates of rapid viral response and, in some cases, sustained viral response amongst HCV infected patients. In each case, Phase II/III data are now emerging.

Financial & competing interests disclosure

Our laboratory actively collaborates with Inhibitex inc., whose agent INX-189 is discussed herein, and we receive financial support for this work, including milestones on progression. C McGuigan is a Board member of Inhibitex Inc. and holds stock and options in the company. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
■ of interest

- Schiff ER. Diagnosis and treating hepatitis C virus infection. *Am. J. Manag. Care* 17(Suppl. 4), S108–S115 (2011).
- Giovanni M, Joanne ET, Steven SC *et al.* Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. *J. Biol. Chem.* 278, 49164–49170 (2003).
- **Focuses on important aspects of drug resistance in hepatitis C virus (HCV) treatment.**
- Hepatitis C – global prevalence (update). *Wkly Epidemiol. Rec.* 75, 18–19 (2000).
- Liang TJ, Reherman B, Seeff LB, Hoofnagle JH. Pathogenesis, natural history, treatment, and prevention of hepatitis C. *Annu. Intern. Med.* 132, 296–305 (2000).
- Thomas DL, Seeff LB. Natural history of hepatitis C. *Clin. Liver Dis.* 9, 383–398 (2003).
- Chun IY, Bor-Luen C. A new insight into hepatitis C vaccine development. *J. Biomed. Biotech.* 548280, 1–12 (2010).
- Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244, 359–362 (1989).
- Freeman AJ, Marinos G, Ffrench RA, Lloyd AR. Immunopathogenesis of hepatitis C virus infection. *Immunol. Cell Biol.* 79, 515–536 (2001).
- Burke KP, Cox AL. A hepatitis C virus evasion of adaptive immune response – a model for viral persistence. *Immunol. Res.* 47, 216–227 (2010).
- Neumann AU, Lam NP, Dahari H, Gretch DR, Layden TJ, Perelson AS. Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon- α therapy. *Science* 282, 103–107 (1998).
- Smith DB, Pathirana S, Davidson F *et al.* The origin of hepatitis C virus genotypes. *J. Gen. Virol.* 78, 321–328 (1997).
- Lee CM, Hung CH, Lu SN, Changchien CS. Hepatitis C virus genotypes: clinical relevance and therapeutic implications. *Chang Gung Med. J.* 31, 16–24 (2008).
- Soriano V, Peters MG, Zeuzem S. New therapies for hepatitis C virus infection. *Clin. Infect. Dis.* 48, 313–320 (2009).
- Zeuzem S, Berg T, Moeller B *et al.* Expert opinion on the treatment of patients with chronic hepatitis C. *J. Viral Hepat.* 16, 75–90 (2009).
- Sarasin-Filipowicz M. Interferon therapy of hepatitis C: molecular insights into success and failure. *Swiss Med. Wkly* 140, 3–11 (2010).
- Pearlman BL, Traub N. Sustained virological response to antiviral therapy for chronic hepatitis C virus infection: a cure and so much more. *Clin. Infect. Dis.* 52, 889–900 (2011).
- Fried MW, Shiffman ML, Reddy KR *et al.* PEGinterferon alfa-2a plus ribavirin for chronic hepatitis C infection. *N. Engl. J. Med.* 347, 975–982 (2002).
- Aspinall RJ, Pockros PJ. The management of side effects during therapy for hepatitis C. *APT* 20, 917–929 (2004).
- Aurora R, Donlin MJ, Cannon NA, Travis JE. Genome-wide hepatitis C virus amino acid covariance networks can predict response to antiviral therapy in humans. *J. Clin. Invest.* 119, 225–236 (2009).
- Asselah T, Benhamou Y, Marcellin P. Protease and polymerase inhibitors for the treatment of hepatitis C. *Liver Intl* 29, 57–67 (2009).
- Simmonds P. Genetic diversity and evaluation of hepatitis C virus – 15 years on. *J. Gen. Virol.* 85, 3173–3188 (2004).
- Ago H, Adachi T, Yoshida A *et al.* Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Cell* 11, 1417–1426 (1999).
- Lesburg CA, Cable MB, Ferrari E *et al.* Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat. Struct. Biol.* 6, 937–943 (1999).
- Ranjith-Kumar CT, Gutshall L, Sarisky RT, Kao CC. Multiple interactions within the hepatitis C virus RNA polymerase represses primer-dependent RNA synthesis. *J. Mol. Biol.* 330, 675–685 (2003).
- Harrus D, El-Sayed NA, Simister PC *et al.* Further insights into the roles of GTP and C-terminus of hepatitis C virus polymerase in the initiation of RNA synthesis. *J. Biol. Chem.* 285, 32906–32918 (2010).
- Furman PA, Lam AM, Murakami E. Nucleoside analogue inhibitors of hepatitis C viral replication: recent advances, challenges and trends. *Future Med. Chem.* 1, 1429–1452 (2009).
- Betzi S, Eydoux C, Bussetta C *et al.* Identification of allosteric inhibitors blocking the hepatitis C virus polymerase NS5B in the RNA synthesis initiation step. *Antiviral Res.* 84, 48–59 (2009).
- Tomei L, Altamura S, Bartholomew L *et al.* Characterization of inhibition of hepatitis C virus RNA replication by nonnucleosides. *J. Virol.* 78, 938–946 (2004).
- Summa V, Petrocchi A, Pace P *et al.* Discovery of alpha, gamma-diketo acids as potent selective and reversible inhibitors of hepatitis C virus NS5b RNA-dependent RNA polymerase. *J. Med. Chem.* 47, 14–17 (2004).
- He Y, Duan W, Tan SL. Emerging host cell targets for hepatitis C therapy. *Drug Discov. Today* 12, 209–217 (2007).
- Tomei A, Altamura S, Paonessa G, De Francesco R, Migliaccio G. HCV antiviral resistance: the impact of *in vitro* studies on the development of antiviral agents targeting the viral NS5B polymerase. *Antivir. Chem. Chemother.* 16, 225–245 (2005).
- Shim J, Larson G, Lai V, Naim S, Wu JZ. Canonical 3'-deoxyribonucleotides as a chain terminator for HCV NS5B RNA-dependent RNA polymerase. *Antivir. Res.* 58, 243–251 (2003).
- Gallois-Montbrun S, Chen Y, Dutartre H *et al.* Structural analysis of the activation of ribavirin analogs by NDP kinase: comparison with other ribavirin targets. *Mol. Pharmacol.* 63, 538–546 (2003).
- De Clercq E. The design of drugs for HIV and HCV. *Nat. Rev. Drug Discov.* 6, 1001–1018 (2006).
- Sofia MJ. Nucleotide prodrugs for HCV therapy. *Antivir. Chem. Chemother.* 22, 23–49 (2011).
- **Provides an important overview of anti-HCV prodrugs.**
- Boback DR, Schinazi RF, Coats SJ. Advances in nucleoside monophosphate prodrugs as anti-HCV agents. *Antivir. Ther.* 15, 935–950 (2010).
- Brown NA. Progress towards improving antiviral therapy for hepatitis C with

- hepatitis C virus polymerase inhibitors. Part I: nucleoside analogues. *Expert Opin. Invest. Drugs* 18, 709–725 (2009).
- Provides a good overview of anti-HCV prodrugs and highlights problems of drug resistance.
- 38 Carroll SS, Olsen DB. Nucleoside analog inhibitors of hepatitis C viral replication. *Infect. Disord. Drug Target.* 6, 17–29 (2009).
- 39 Pierra C, Amador A, Benzaria S *et al.* Synthesis and pharmacokinetics of Valopicitabine (NM283), an efficient prodrug of the potent anti-HCV agent 2'-C-Methylcytidine. *J. Med. Chem.* 49, 6614–6620 (2006).
- 40 Le Pogam S, Jiang WR, Leveque V *et al.* *In vitro* selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. *Virology* 351, 349–359 (2006).
- 41 Ali S, Leveque V, LePogam S *et al.* Selected replicon variants with low-level *in vitro* resistance to the hepatitis C virus NS5B polymerase inhibitors PSI6130 lack cross-resistance with R1479. *Antimicrob. Agents Chemother.* 52, 4356–4369 (2008).
- 42 Bichko V, Lallous L, Soubasakos M *et al.* Valopicitabine (NM 283) is fully active against known HCV protease resistance mutations *in vitro*. *J. Hepat.* 46(Suppl. 1), S163 (2007).
- 43 Sherman KE, Lawitz E, O'Brien CB, Godofsky E, Brown NA. Valopicitabine (NM283) plus PEG-interferon for chronic hepatitis C in treatment naïve and nonresponders patients: interim Phase IIb results. *J. Clin. Virol.* 36(Suppl. 2), S24 (2006).
- 44 Kwong AD, McNair L, Jacobson I, George S. Recent progress in the development of selective hepatitis C virus NS3*4A protease and NS5B polymerase inhibitors. *Curr. Opin. Pharmacol.* 8, 522–531 (2008).
- 45 Smith DB, Martin JA, Klumpp K *et al.* Design, synthesis, and antiviral properties of 4'-substituted ribonucleosides as inhibitors of hepatitis C virus replication: the discovery of R1479. *Bioorg. Med. Chem. Lett.* 17, 2570–2576 (2007).
- 46 Roberts SK, Cooksley G, Dore GJ *et al.* Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. *Hepatology* 48, 398–406 (2008).
- 47 Pockros PJ, Nelson D, Godofsky E *et al.* R1626 plus PEG-interferon alfa-2a provides potent suppression of hepatitis C virus RNA and significant antiviral synergy in combination with ribavirin. *Hepatology* 48, 385–397 (2008).
- 48 Stuyver LJ, McBrayer TR, Tharnish PM *et al.* Inhibition of hepatitis C replicon RNA synthesis by β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine: a specific inhibitor of hepatitis C virus replication. *Antivir. Chem. Chemother.* 17, 79–87 (2006).
- 49 Murakami E, Bao H, Ramesh M *et al.* Mechanism of activation of β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine and inhibition of hepatitis C virus NS5B RNA polymerase. *Antimicrob. Agents Chemother.* 51, 503–509 (2007).
- 50 Ma H, Jiang WR, Robledo N *et al.* Characterization of the metabolic activation of hepatitis C virus nucleoside inhibitor β -D-2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI6130) and identification of novel active 5'-triphosphate species. *J. Biol. Chem.* 282, 29812–29820 (2007).
- 51 McHutchison JG, Reddy R, Rodriguez-Torres M *et al.* Potent antiviral activity of the nucleoside HCV inhibitor, R7128, in prior IFN nonresponders. Presented at: *Frontiers in Drug Development in Viral Hepatitis (HEP-DART)*. Lahaina, Hawaii, USA, 9–13 December 2007.
- 52 Hecker SJ, Erion MD. Prodrugs of phosphonates and phosphates. *J. Med. Chem.* 51, 2328–2345 (2008).
- 53 Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Nat. Leukemia* 15, 875–890 (2001).
- 54 Mackman RL, Cihlar T. Prodrug strategies in the design of nucleoside and nucleotide antiviral therapeutics. *Annu. Rev. Med. Chem.* 39, 305–321 (2004).
- 55 Ariza ME. Current prodrug strategies for the delivery of nucleotides into cells. *Drug Des. Rev. Online* 2, 373–387 (2005).
- 56 Mehellou Y, Balzarini J, McGuigan C. Aryloxyphosphoramidates triesters: a technology for delivering monophosphorylated nucleosides and sugars into cells. *ChemMedChem* 4, 1779–1791 (2009).
- 57 Zemlicka J. Lipophilic phosphoramidates as antiviral pronucleotides. *Biochim. Biophys. Acta* 1587, 276–286 (2002).
- 58 Pojarvi-Virta P. Prodrug approaches of nucleotides and oligonucleotides. *Curr. Med. Chem.* 13, 3441–3465 (2006).
- 59 McGuigan C, Pathirana RN, Mahmood N, Devine GK, Hay AJ. Aryl phosphate derivatives of AZT retain activity against HIV1 in cell lines, which are resistant to the action of AZT. *Antivir. Res.* 17, 311–321 (1992).
- 60 Devine KG, McGuigan C, O'Connor TJ, Nicholls SR, Kinchington D. Novel phosphate derivatives of zidovudine as anti-HIV compounds. *AIDS* 4, 371–372 (1990).
- 61 McGuigan C, Devine KG, O'Connor TJ, Galpin SA, Jeffries DJ, Kinchington D. Synthesis and evaluation of some novel phosphoramidate derivatives of 3'-azido-3'-deoxythymidine (AZT) as anti-HIV compounds. *Antivir. Chem. Chemother.* 1, 107–113 (1990).
- 62 McGuigan C, Devine K, O'Connor TJ, Kinchington D. Synthesis and anti-HIV activity of some haloalkyl phosphoramidate derivatives of 3'-azido-3'-deoxythymidine (AZT): potent activity of the trichloroethyl methoxyalaninyl compound. *Antiviral Res.* 15, 255–263 (1991).
- 63 Balzarini J, Kruijning J, Wedgwood O *et al.* Conversion of 2',3'-dideoxyadenosine (ddA) and 2',3'-didehydro-2',3'-dideoxyadenosine (d4A) to their corresponding aryloxyphosphoramidate derivatives markedly potentiates their activity against human immunodeficiency virus and hepatitis B virus. *FEBS Lett.* 410, 324–328 (1997).
- 64 Perrone P, Luoni GM, Kelleher MR *et al.* Application of the phosphoramidate protide approach to 4'-azidouridine confers sub-micromolar potency versus hepatitis C virus on an inactive nucleoside. *J. Med. Chem.* 50, 1840–1849 (2007).
- 65 Perrone P, Daverio F, Valente R *et al.* First example of phosphoramidate approach applied to a 4'-substituted purine nucleoside (4'-azidoadenosine): conversion of an inactive nucleoside to a submicromolar compound versus hepatitis C virus. *J. Med. Chem.* 50, 5463–5470 (2007).
- 66 McGuigan C, Cahard D, Sheeka HM, De Clercq E, Balzarini J. Aryl phosphoramidate derivatives of d4T have improved anti-HIV efficacy in tissue culture and may act by the generation of novel intracellular metabolites. *J. Med. Chem.* 39, 1748–1753 (1996).
- 67 Balzarini J, Karlsson A, Aquaro S *et al.* Mechanism of anti-HIV action of masked alaninyl D4TMP derivatives. *Proc. Natl Acad. Sci. USA* 93, 7295–7299 (1996).
- 68 Ballatore C, McGuigan C, De Clercq E, Balzarini J. Synthesis and evaluation of novel amidate prodrugs of PMEA and PMPA. *Bioorg. Med. Chem. Lett.* 11, 1053–1056 (2001).
- 69 McGuigan C, Harris SH, Daluge SM *et al.* Application of phosphoramidate pronucleotide technology to abacavir leads to a significant enhancement of antiviral potency. *J. Med. Chem.* 48, 3504–3515 (2005).
- 70 McGuigan C, Hassan-Abdallah A, Srinivasan S *et al.* Application of phosphoramidate

- ProTide technology significantly improves antiviral potency of carbocyclic adenosine derivatives. *J. Med. Chem.* 49, 7215–7226 (2006).
- 71 McGuigan C, Serpi M, Bibbo R *et al.* Phosphate prodrugs derived from *N*-acetylglucosamine have enhanced chondroprotective activity in explant cultures and represent a new lead in antiosteoarthritis drug discover. *J. Med. Chem.* 51, 5807–5812 (2008).
- 72 McGuigan C, Tsang HW, Sutton PW, De Clercq E, Balzarini J. Synthesis and anti-HIV activity of some novel chain-extended phosphoramidate derivatives of d4T (stavudine): esterase hydrolysis as a rapid predictive test for antiviral potency. *Antiviral Chem. Chemother.* 9, 109–115 (1998).
- 73 McGuigan C, Sutton PW, Cahard D *et al.* Synthesis, anti-human immunodeficiency virus activity and esterase lability of some novel carboxylic ester-modified phosphoramidate derivatives of stavudine (d4T). *Antiviral Chem. Chemother.* 9, 473–479 (1998).
- 74 Abraham TW, Kalman TI, McIntee EJ, Wagner CR. Synthesis and biological activity of aromatic amino acid phosphoramidates of 5'-fluoro-2'-deoxyuridine and 1- β -arabinofuranosylcytosine: evidence of phosphoramidase activity. *J. Med. Chem.* 39, 4569–4575 (1996).
- 75 Kim J, Chou TF, Grieger GW, Wagner CR. Direct measurement of nucleoside monophosphate delivery from a phosphoramidate pronucleotide by stable isotope labeling and LC-ESI MS/MS. *Mol. Pharm.* 1, 102–111 (2004).
- 76 Iyer VV, Grieshaber GW, Radmer MR, McIntee EJ, Wagner CR. Synthesis, *in vitro* anti-breast cancer activity, and intracellular decomposition of amino acid methyl ester alkyl amide phosphoramidate monoesters of 3'-azido-3'-deoxythymidine (AZT). *J. Med. Chem.* 43, 2266–2274 (2000).
- 77 Gosselin G, Girardet JL, Perigaud C *et al.* New insights regarding the potential of the pronucleotide approach in antiviral chemotherapy. *Acta Biochim. Polym.* 43, 195–208 (1996).
- 78 Lefebvre I, Perigaud C, Pompon A *et al.* Mononucleoside phosphotriester derivatives with *S*-acyl-2-thioethyl Bioreversible phosphate-protecting groups: intracellular delivery of 3'-azido-2',3'-dideoxythymidine 5'-monophosphate. *J. Med. Chem.* 38, 3941–3950 (1995).
- 79 Perigaud C, Gosselin G, Lefebvre I *et al.* Rational design for cytosolic delivery of nucleoside monophosphates: SATE and DTE as enzyme labile transient phosphate protecting groups. *Bioorg. Med. Chem. Lett.* 3, 2521–2526 (1993).
- 80 Valette G, Pompon A, Girardet JL *et al.* Decomposition pathways and *in vitro* HIV inhibitory effects of isoddA pronucleotides: toward a rational approach for intracellular delivery of nucleoside 5'-monophosphates. *J. Med. Chem.* 39, 1981–1990 (1996).
- 81 Phavc M, Dyatkina N, Keicher J *et al.* Synthesis and biological activity of 7-deaza-7-ethynyl-2'-deoxy-2'-*C*-methyladenosine and its 2'-*C*-Methyl-*ribo* analogue. *Nucleic Acids Symp. Ser.* 52, 643–644 (2008).
- 82 Bezaria S, Bardiot D, Bouisset T *et al.* 2'-*C*-Methyl branched pyrimidine ribonucleoside analogues: potent inhibitors of RNA virus replication. *Antivir. Chem. Chemother.* 18, 225–242 (2007).
- 83 Gunic E, Girardet JL, Ramasamy K *et al.* Cyclic monophosphate prodrugs of base-modified 2'-*C*-methyl ribonucleosides as potent inhibitors of hepatitis C virus RNA replication. *Bioorg. Med. Chem. Lett.* 17, 2452–2455 (2007).
- 84 Zhou XJ, Pietropaolo K, Chen J *et al.* Safety and pharmacokinetics of IDX184, a liver-targeted nucleoside polymerase inhibitor of hepatitis C virus, in healthy subjects. *Antimicrob. Chem. Chemother.* 55, 76–81 (2011).
- 85 LePage GA, Hersh EM. Cyclic nucleotide analogs as carcinostat agents. *Biochem. Biophys. Res. Commun.* 46, 1918 (1972).
- 86 Gohil RN, Gillen RG, Nagyvary J. Synthesis and properties of some cyclic AMP alkyl phosphotriesters. *Nucleic Acids Res.* 12, 1691–1702 (1974).
- 87 Meyer RB, Stone TE. 2'-*O*-Acyl-6-thioinosine cyclic 3',5'-phosphates as prodrugs of thioinosinic acid. *J. Med. Chem.* 22, 811–815 (1979).
- 88 Engels J, Schlaeger EJ. Synthesis, structure, and reactivity of adenosine cyclic 3',5'-phosphate benzyl triesters. *J. Med. Chem.* 20, 907–911 (1977).
- 89 Reddy PG, Bao D, Chang W *et al.* 2'-deoxy-2'- α -fluoro-2'- β -*C*-methyl 3',5'-cyclic phosphate nucleotide prodrug analogs as inhibitors of HCV NS5B polymerase: discovery of PSI352938. *Bioorg. Med. Chem. Lett.* 24, 7376–7380 (2010).
- 90 Meppen M, Pacini B, Bazzo R *et al.* Cyclic phosphoramidates as prodrugs of 2'-*C*-methylcytidine. *Eur. J. Med. Chem.* 44, 3765–3770 (2009).
- 91 Erion MD, Reddy RK, Boyer SH *et al.* Design, synthesis and characterization of a series of cytochrome P450 3A-activated prodrugs (HepDirect prodrugs) useful for targeting phosph(on)ate-based drugs to the liver. *J. Am. Chem. Soc.* 126, 5154–5163 (2004).
- 92 Erion MD, van Poelje PD, Mackenna DA *et al.* Liver-targeted drug delivery using HepDirect prodrugs. *J. Pharmacol. Exp. Ther.* 312, 554–560 (2005).
- 93 Sorbera LA, Serradell N, Bolos J. Pradefovir mesilate treatment for hepatitis B. *Drugs Future* 32, 137–143 (2007).
- 94 Gardelli C, Attenni B, Donghi M *et al.* Phosphoramidate prodrugs of 2'-*C*-methylcytidine for therapy of hepatitis C virus infection. *J. Med. Chem.* 52, 5394–5407 (2009).
- 95 Carroll SS, Koeplinger K, Vavrek M *et al.* Antiviral efficacy upon administration of HepDirect prodrug of 2'-*C*-methylcytidine to hepatitis C virus-infected chimpanzees. *Antimicrob. Agents Chemother.* 55, 3854–3860 (2011).
- 96 Murakami E, Niu C, Bao H *et al.* The mechanism of action of beta-D-2'-deoxy-2'-fluoro-2'-*C*-methylcytidine involves a second metabolic pathway leading to beta-D-2'-deoxy-2'-fluoro-2'-*C*-methyluridine 5'-triphosphate, a potent inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. *Antimicrob. Agents Chemother.* 52, 458–564 (2008).
- 97 Sofia MJ, Bao D, Chang W *et al.* Discovery of β -D-2'-deoxy-2'-fluoro-2'-*C*-methyluridine nucleotide prodrug (PSI7977) for the treatment of hepatitis C virus. *J. Med. Chem.* 53, 7202–7218 (2010).
- 98 Pharmasset reports positive preliminary antiviral data with Psi-7851 for the treatment of hepatitis C. *Pharmasset* 31 July 2009.
- 99 Murakami E, Tolstykh T, Bao H *et al.* Mechanism of activation of PSI7851 and its diastereoisomer PSI7977. *J. Biol. Chem.* 285, 34337–34347 (2010).
- 100 Lawitz E, Lalezari J, Rodriguez-Torres M *et al.* High rapid virological response (RVR) with PSI7977 daily dosing plus PEG-IFN/ RBV in a 28-day Phase 2a trial. Presented at: *61st Annual Meeting of the American Association of the Study of Liver Diseases*. Boston, MA, USA, 29 October–2 November 2010.
- 101 Lawitz E, Rodriguez-Torres M, Denning J *et al.* Once daily dual nucleotide combination of PSI938 and PSI7977 provides 94% HCV RNA <LOD at day 14: first purine/ pyrimidine clinical combination data (the nuclear study). Presented at: *46th Annual Meeting of the European Association for the Study of the Liver, EASL*. Berlin, Germany, 30 March–3 April 2011.

- 102 McGuigan C, Kelleher MR, Perrone P *et al.* The application of phosphoramidate ProTide technology to the potent anti-HCV compound 4'-azidocytidine (R1479). *Bioorg. Med. Chem. Lett.* 19, 4250–4254 (2009)
- 103 Perrone P, Luoni GM, Keheller MR *et al.* Application of the phosphoramidate ProTide approach to 4'-azidouridine confers sub-micromolar potency versus hepatitis C virus on an inactive nucleoside. *J. Med. Chem.* 50, 1840–1849 (2007)
- 104 Carroll SS, Tomassini JE, Bosserman M *et al.* Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. *J. Biol. Chem.* 278, 11979–11984 (2003).
- 105 McGuigan C, Perrone P, Madala K *et al.* The phosphoramidate ProTide approach greatly enhances the activity of β -2'-C-methylguanosine against hepatitis C virus. *Bioorg. Med. Chem. Lett.* 19, 4316–4320 (2009).
- 106 Hecker SJ, Reddy KR, van Poelje PD *et al.* Liver-targeted prodrugs of 2'-C-methyladenosine for therapy of hepatitis C virus infection. *J. Med. Chem.* 50, 3891–3896 (2007).
- 107 Carroll SS, Ludmerer S, Handt L *et al.* Robust antiviral efficacy upon administration of nucleoside analog to hepatitis C virus-infected chimpanzees. *Antimicrob. Agents Chemother.* 53, 926–934 (2009).
- 108 Cretton-Scott E, Periguad C, Peyrottes S *et al.* In vitro antiviral activity and pharmacology of IDX184, a novel and potent inhibitor of HCV replication. *J. Hepatol.* 18(Suppl. 2), S220 (2008).
- 109 Lalloo L, LaColla M, Serra I *et al.* Combination of IDX184, a nucleotide prodrug polymerase inhibitor with other classes of HCV inhibitors is additive to synergistic in the HCV replicon *in vitro*. Presented at: *60th Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 30 October–3 November 2009.
- 110 Standring D, Lanford R, Li B *et al.* Antiviral activity of the liver targeted nucleoside HCV polymerase inhibitor IDX184 correlates with trough serum levels of the nucleoside metabolite of HCV-infected chimpanzees. Presented at: *44th Annual Meeting of the European Association for the Study of the Liver, EASL*. Copenhagen, Denmark, 22–26 April 2009.
- 111 Lalezari J, Asmuth D, Casiro A *et al.* Antiviral activity, safety and pharmacokinetics of IDX184, a liver-targeted nucleotide HCV polymerase inhibitor, in patients with chronic hepatitis C. Presented at: *60th Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 30 October–3 November 2009.
- 112 Lalezari J, O'Riordan W, Poordad F *et al.* A Phase IIa study of IDX184 in combination with PEGylated interferon (PEG-IFN) and ribavirin (RBV) in treatment-naïve HCV genotype 1-infected subjects. Presented at: *61st Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 29 October–2 November 2010.
- 113 McGuigan C, Gilles A, Madala K *et al.* Phosphoramidate ProTides of 2'-C-Methylguanosine as highly potent inhibitors of hepatitis C virus. Study of their *in vitro* and *in vivo* properties. *J. Med. Chem.* 53, 4949–4957 (2010)
- 114 McGuigan C, Madala K, Aljarah M *et al.* Design, synthesis and evaluation of a novel double prodrug: INX08189. A new clinical candidate for hepatitis C virus. *Bioorg. Med. Chem. Lett.* 20, 4850–4854 (2010)
- 115 McGuigan C, Madala K, Aljarah M *et al.* Dual pro-drugs of 2'-C-methyl guanosine monophosphate as potent and selective inhibitors of hepatitis C virus. *Bioorg. Med. Chem. Lett.* 21, 6007–6012 (2011).
- 116 Vernachio JH, Blaiman B, Bryant KD *et al.* INX08189, a phosphoramidate prodrug of 6-O-methyl-2'-C-methyl guanosine, is a potent inhibitor of hepatitis C virus replication with excellent pharmacokinetic and pharmacodynamic properties. *Antimicrob. Agents Chemother.* 55, 1843–1851 (2011).
- 117 Hall A, Chamberlain S, Ganguly B *et al.* Metabolite Characterization of INX189, a potent HCV inhibitor, in fresh human primary hepatocytes and human liver cell lines. Presented at: *61st Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 29 October–2 November 2010.
- 118 Barry A, Patti J, Matson M *et al.* A study of the safety and pharmacokinetics of single ascending oral dose of INX08189, a nucleotide polymerase inhibitor, in healthy subjects. Presented at: *46th Annual Meeting of the European Association for the Study of the Liver, EASL*. Berlin, Germany, 30 March–3 April 2011.
- 119 Chang W, Bao D, Chun BK *et al.* Discovery of PSI353661, a novel purine nucleotide prodrug for the treatment of HCV infection. *ACS Med. Chem. Lett.* 2, 130–135 (2010)
- 120 Reddy PG, Bao D, Chang W *et al.* 2'-Deoxy-2'- α -fluor-2'- β -C-methyl 3',5'-cyclic phosphate nucleotide prodrug analogs as inhibitors of HCV NS5B polymerase: discovery of PSI352938. *Bioorg. Med. Chem. Lett.* 20, 7376–7380 (2010)
- 121 Symonds WT, Denning JM, Albanis E *et al.* Pharmacokinetics, safety and tolerability of PSI352938, a novel nucleotide polymerase inhibitor for HCV, following single ascending oral doses in healthy subjects. Presented at: *61st Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 29 October–2 November 2010.
- 122 Furman PA, Murakami E, Niu C. Activity and the metabolic activation pathway of the potent and selective hepatitis C virus pronucleotide inhibitor PSI353661. *Antivir. Res.* 91, 120–132 (2011)
- 123 Gunic E, Chow S, Rong F *et al.* 6-Hydrazinopurine 2'-methyl ribonucleosides and their 5'-monophosphate prodrugs as potent hepatitis C virus inhibitors. *Bioorg. Med. Chem. Lett.* 17, 2456–2458 (2007).
- 124 Zhou Y, Muh U, Hanzelka BL *et al.* Phenotypic and structural analyses of hepatitis C virus NS3 protease Arg155 variants: sensitivity to telaprevir (VX0950) and interferon alpha. *J. Biol. Chem.* 282, 22619–22628 (2007).
- 125 Le Pogam S, Sessaadri A, Kosaka A *et al.* Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients. *J. Antimicrob. Ther.* 61, 1205–1216 (2008).
- 126 Le Pogam S, Sessaadri A, Kang H *et al.* Low levels of resistance, low viral fitness and absence of resistant mutations in baseline quasispecies may contribute to high barrier to R1626 resistance *in vivo*. *J. Hepatol.* 48(Suppl. 2), S10 (2008).
- 127 McCown MF, Rajyaguru S, Le Pogam *et al.* The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. *Antimicrob. Agents Chemother.* 52, 1604–1612 (2008).
- 128 Ali S, Leveque V, Le Pogam S *et al.* Selected replicon variants with low-level *in vitro* resistance to the hepatitis C virus NS5B polymerase inhibitor PSI6130 lack cross-resistance with R1479. *Antimicrob. Agents Chemother.* 52, 4356–4369 (2008).
- 129 Cheng G, Fenzux M, Mabery E *et al.* Nucleotide inhibitor resistance selections using GT2A infectious virus: PSI7851 and GS-6620 select for novel resistance pathway including substitutions of M289V/L followed by S282T. Presented at: *46th Annual Meeting of the European Association for the Study of the Liver, EASL*. Berlin, Germany, March 30–3 April 2011.

- 130 Lam AM, Espiritu C, Micolochick Steuer H *et al.* Novel 2'-F-2'-C-methylpurine nucleotide analogs are active inhibitors of HCV replication and lack cross-resistance with other nucleos(t)ide analogs. Presented at: *61st Annual Meeting of the American Association for the Study of Liver Diseases*. Boston, MA, USA, 29 October–2 November 2010.
- 131 Traut TW. Physiological concentrations of purines and pyrimidines. *Mol. Cell. Biochem.* 140, 1–22 (1994).

■ Patent

- 201 American Cyanamid Company: US07/839728.

■ Websites

- 301 Roche.
www.roche.com/investors/ir_update/inv-update-2008-2010-21.html
- 302 Pharmasset.
www.pharmasset.com/pipeline/rg7128.aspx
- 303 Pharmasset.
www.pharmasset.com/pipeline/psi_7851.aspx
- 304 Pharmasset.
www.pharmasset.com/assets/1/workflow_staging/AssetManager/115.PDF
- 305 Pharmasset
http://files.shareholder.com/downloads/VRUS/1352603825x0x434604/59c80bad-fe3f-4f52-8e25-47fdac6bddf4/VRUS_News_2011_1_10_General_Releases.pdf
- 306 Pharmasset
http://files.shareholder.com/downloads/VRUS/1352603825x0x480698/beab5196-b678-4466-84a6-62b4f56efcba/VRUS_News_2011_7_6_General_Release.pdf
- 307 HIV and Hepatitis.com.
www.hivandhepatitis.com/hep_c/news/2010/0910_2010_b.html
- 308 Idenix Pharmaceuticals.
<http://phx.corporate-ir.net/External.File?item=UGFyZW50SUQ9NDM5NTA0fENoaWxkSUQ9NDYxMDMxfFR5cGU9MQ=&t=1>
- 309 Inhibitex.
www.inhibitex.com/Pipeline/Hepatitis.html
- 310 InvestorsHub.
http://investorshub.advn.com/boards/read_msg.aspx?message_id=56063911